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(54) Title: BENZAMIDE INHIBITORS OF BACTERICAL LIPOPROTEIN SIGNAL PEPTIDASE

(57) Abstract: Increasing resistance to antibiotics necessitates discovery of new targets and strategies to combat bacteria. Ideal protein targets are required for viability across many species, are unique to prokaryotes to limit effects on the host and have robust assays to quantitate activity and identify novel inhibitors. Lipoprotein signal peptidase (Lsp) is a transmembrane aspartyl protease required for lipoprotein maturation and entirely fits these criteria. We have developed the first in vitro high-throughput assay to monitor proteolysis by Lsp. We employed our HTS assay against 646,275 compounds to discover inhibitors of Lsp and synthesized a range of analogues to generate molecules with nanomolar IC50 values. Importantly, our inhibitors are effective in preventing the growth of E. coli cultures. Our Lsp assay will be a useful tool for biologists to monitor Lsp activity and our inhibitors will facilitate development of antibacterial agents to potentially treat antibiotic-resistant bacteria.

Benzamide Inhibitors of Bacterial Lipoprotein Signal Peptidase

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority of U.S. provisional application Ser. No. 62/528,759, filed July 5, 2017.

BACKGROUND

Lipoprotein signal peptidase (Lsp, also known as SPaseII) is an aspartic acid protease with a pivotal role in bacterial lipoprotein maturation.[1-3] Lipoproteins have an array of important roles in bacteria that include, but are not limited to, nutrient uptake, adhesion, sporulation, protein transport, secretion, small molecule export, cell wall biosynthesis, and antibiotic resistance.[4-6] During the lipoprotein maturation process, Lsp recognizes a region of the signal sequence within the prolipoprotein termed the lipobox and removes all residues N-terminal to a post-translationally modified diacylglyceryl (DAG)-cysteine residue (Figure 1A).[7] Lsp is an attractive target for inhibitor discovery for several reasons, including: 1) most bacteria possess a single *lsp* gene;[5] 2) the Lsp sequence and structure is conserved across bacteria; 3) organisms other than bacteria do not have Lsp homologues thus the inhibitor could have high selectivity to bacteria; and 4) the unique mechanism-of-action by Lsp inhibitors should overcome drug-resistant bacteria and may synergistically promote the efficacy of current antibiotics. The x-ray structure of Lsp from *Pseudomonas aeruginosa* was recently determined and showed that Lsp is a monomer consisting of four transmembrane helices with an active site that lies within the lipid bilayer.[8]

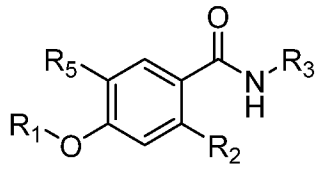
Lsp has been considered as a promising target for the design of new classes of antibiotics for decades, based on the observation that two natural product Lsp inhibitors, globomycin and myxovirescin, have antibacterial efficacy.[9-14] Since their discovery in 1978 from *Streptomyces halstedii*, the hydrophobic cyclic peptide globomycin and synthetic analogues have demonstrated potent activity against bacterial cultures.[9,15] A more recent study suggested that another natural product synthesized by *Myxococcus xanthus*, termed myxovirescin, inhibited Lsp based on the results of a whole cell assay.[14,16] Unfortunately, the limited stability, *in vivo* ineffectiveness, scalability, and accessibility currently render these molecules as intractable.[17] Additional Lsp inhibitors with novel mechanisms of action have yet to

be identified and the dearth of molecules is primarily due to the lack of a robust and high-throughput *in vitro* assay.

SUMMARY

The invention is directed, in various embodiments, to compounds that are effective inhibitors of a bacterial lipoprotein signal peptidase, to methods of identifying such inhibitors using a FRET analysis suitable for high-throughput screening, to methods of inhibiting a bacterial lipoprotein signal peptidase, and to methods of treatment of bacterial infections in patients.

The invention provides, in various embodiments, a compound of formula (1)



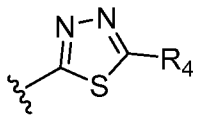
(1),

wherein

R_1 is (C1-C6) alkyl, or is a 5-, 6- or 7-membered cycloalkyl;

R_2 is H, NO_2, halo, or trifluoromethyl;

R_3 is a 1,3,4-thiadiazole of formula



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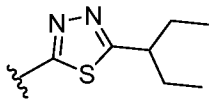
wherein R_4 is (C4-C6) straight or branched chain alkyl, or is a 5-, 6- or 7-membered cycloalkyl;

R_5 is H or (C1-C4)alkoxyl;

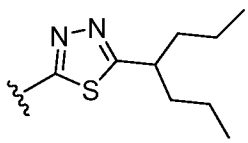
or a pharmaceutically acceptable salt thereof.

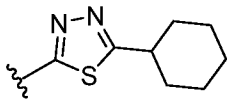
For example, R_1 can be isopropyl or cyclopentyl. For example, R_2 can be NO_2.

More specifically, the 1,3,4-thiadiazole R_3 can be any one of

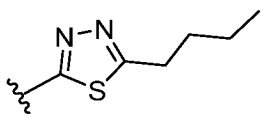


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, or



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In other embodiments, the invention provides a method of inhibiting a bacterial lipoprotein signal peptidase (Lsp), comprising contacting the peptidase with an effective amount or concentration of a compound of the invention as described herein.

The invention further provides a method of treatment of a bacterial infection in a patient, comprising administering to the patient an effective dose of a compound of the invention, as described herein.

In various embodiments, the invention also provides a method of screening a compound for inhibitory bioactivity of a bacterial lipoprotein signal peptidase (Lsp), comprising contacting a Lsp peptide FRET substrate, comprising a hexapeptide VTGCAK, with a N-terminal dabsyl quencher and C-terminal EDANS fluorophore wherein the cysteine reside of the hexapeptide is S-alkylated with a diacylglycerol residue, and a candidate inhibitor compound, then measuring fluorescence from the fluorophore signalling cleavage of the Lsp FRET substrate and its inhibition by the candidate inhibitor. For example, the screening of multiple compounds can be carried out in parallel in a High Throughput Screening format.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. A) Schematic of Lsp cleavage of lipobox residues (green) preceding the DAG-modified cysteine (red). B) Design strategy for a peptide-based FRET reporter substrate with a N-terminal quencher (blue circle) and C-terminal fluorophore (green circle) to rapidly quantitate Lsp activity *in vitro.*

Figure 2. A) Structure of Lsp peptide FRET substrate with a N-terminal dabsyl quencher (blue) and C-terminal EDANS fluorophore (green). Lsp from *E. coli* requires the DAG-modified cysteine residue (red) for activity. B). Kinetic assays with Lsp from *E. coli.* Michaelis-Menton kinetic constants of the optimized FRET substrate are K_M = 14.2 $\pm$ 4.6 μM and k_{cat} = 0.01 $\pm$ 0.0001 s^{-1}. [Lsp] = 400 nM.

Figure 3. *In vitro* inhibition of *E. coli* Lsp by **1j** (blue), and **1i** (green). [S] = 50 mM, [Lsp] = 100 nM. Mean ± SD values are shown. Hill constants are approximately 1 for both compounds, suggesting that there is no cooperativity. Compounds have no effect on cleaved substrate fluorescence.

Figure 4. Lsp inhibitors in combination with polymyxin B nonapeptide (PMBN) slows the growth of *E. coli*. Mean ± SD values are shown. A) Concentration-response

curves against *E. coli* (ATCC25922) after 16 h incubation. The full time course is shown in Figure S7. The highest concentration of compound **1j** used was 25 mM due to the limited solubility and all assays employed a concentration of PMNB at 8 mg/L. Optical density at 600 nm (OD_{600}) values before the bacterial growth (at 0 h) are shown as 'Background'. B) Lsp-inhibition resistant mutant YX23 is resistant to compound **1j**. Bacteria was treated with PMBN (8 mg/L) or **1j** (25 mM) + PMBN (8 mg/L). OD_{600} values at 10 h or 24 h are shown for DW37 or YX23, respectively, for comparison between strains. Student's *t*-test was used to calculate p-values with < 0.05 considered significant.

Figure 5. The effect of linker chain length of 2-6 versus potency is plotted in relation to the compounds of Table 7.

Figure 6. Concentration-response curves of globomycin on bacteria growth

DETAILED DESCRIPTION

Herein, we report the design of a robust Lsp substrate and *in vitro* assay that provides the first rapid and quantitative method to study Lsp biochemistry and serves as a platform to discover small-molecule inhibitors. See: S. Kitamura, A. Owensby, D. Wall, D. W. Wolan, Cell Chemical Biology 2017, 25, 301-308.e312. (https://www.cell.com/cell-chemical-biology/fulltext/S2451-9456(17)30460-9) and Kitamura, S. and Wolan, D. W. (2018) FEBS Lett. . doi:10.1002/1873-3468.13155 (https://febs.onlinelibrary.wiley.com/doi/abs/10.1002/1873-3468.13155)

We optimized our assay for ultra-high-throughput discovery approaches and screened an in-house library of 646,275 compounds for small molecule inhibitors of Lsp. From this effort, several lead inhibitors were identified, and further medicinal chemistry efforts led to the development of compound **1j** with an IC_{50} of 99 nM. We subjected **1j** to cultures of *E. coli* and demonstrated that the small molecule inhibits bacterial growth.

To interrogate and measure exogenously purified *E. coli* Lsp activity, we synthesized FRET-based peptide substrates consisting of a N-terminal dabsyl quencher and a C-terminal EDANS fluorophore by Fmoc-based solid-phase peptide synthesis (SPPS) (Figure 2A). We found that peptide elongation with DAG-Cys required low-loading capacity resin, and the purification of the peptide required normal phase HPLC using an amide column (detailed in the Supporting Information). Consistent with previous reports,[1] a DAG-Cys modification is essential for substrate recognition in our *in vitro* conditions, as *E. coli* Lsp was unable to hydrolyze FRET

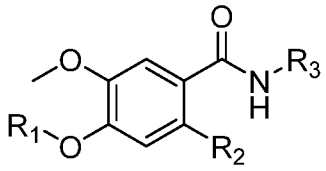
substrates that lacked a lipid tail (data not shown). After optimizing the peptide sequence, DAG structure, and fluorophore pairs, our Lsp substrate contains the peptide sequence -VTG/CAK- and a DAG-modified cysteine with saturated palmitate fatty acids as shown in Figure 2A. Michaelis-Menten kinetic measurements were performed for the hydrolysis of the FRET substrate by Lsp from *E. coli* and has a K_M = 14.2 $\pm$ 4.6 μM and k_{cat} = 0.01 $\pm$ 0.0001 s^{-1} (Figure 2B).

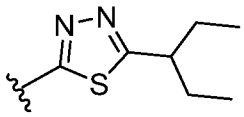
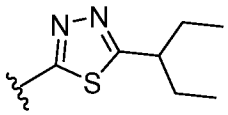
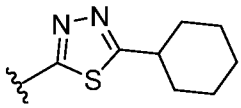
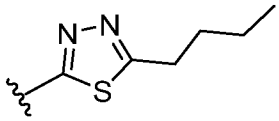
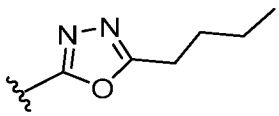
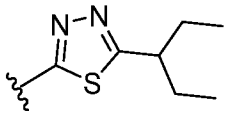
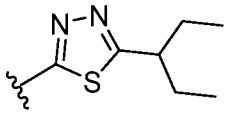
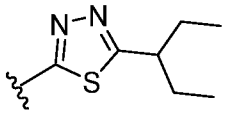
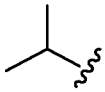
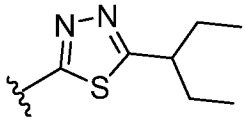
We subsequently validated our optimized assay by determining the inhibitory potency of globomycin. Globomycin inhibited Lsp from *E. coli* with IC_{50} < 5 nM with an enzyme concentration at 10 nM (Figure S1).[3,20] This is consistent with previous reports that globomycin is a potent inhibitor of Lsp.[3,20] In addition, myxovirescin B showed comparable inhibitory potency to globomycin with an IC_{50} < 5 nM (Figure S1). As previously proposed,[2] no known aspartic acid protease inhibitors, including general (pepstatin), γ-secretase (begacestat and semagacestat), renin (aliskiren), and HIV inhibitors (saquinavir, atazaniavir) inhibit *E. coli* Lsp, despite the active site consisting of the canonical two asparatic acid residues responsible for the activation of a water molecule for nucleophilic attack on the substrate peptide backbone carbonyl.[8] To identify new small molecule Lsp inhibitors we therefore performed a high-throughput screen against an in-house compound library.

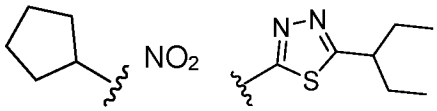
The Lsp FRET assay was miniaturized to 1536-well plates and ultra-high-throughput screening was performed against 646,275 molecules from the Scripps Drug Discovery Library. After eliminating molecules that interfere the fluorescence signal and molecules considered “PAINS” due to promiscuous reactivity,[21-23] we identified several compounds of interest for further study. Based on the potency and synthetic accessibility, a benzamide compound **1a** was chosen for structural optimization. Biochemical analysis revealed that **1a** inhibits Lsp in non-competitive manner. Unlike previous attempts at structure-activity relationships (SAR) with globomycin and myxovirescin, relying on visualization of Braun’s lipoprotein modification by gel-shift assays or MIC determination,[14,15,24] we were able to perform rapid iterative rounds of synthesis and inhibition evaluation with our *in vitro* assay.

The following Tables 1-7 provide structure-activity relationships for various compounds useful for practice of a method of the invention.

Table 1: Structure-activity relationships of benzamide **1a**.



#	R_1	R_2	R_3	IC_{50}[a] (μM)
1a	CH_3	NO_2		4.2 (± 1.0)[b]
1b	CH_3	H		110 (±23)[b]
1c	CH_3	NO_2		>400[b]
1d	CH_3	NO_2		29 (±0.60)[b]
1e	CH_3	NO_2		>400[b]
1f	CH_3CH_2	NO_2		1.2 (±0.10)[b]
1g		NO_2		0.54 (±0.15)[c]
1h		NO_2		0.11 (±0.025)[c]
1i		NO_2		0.21 (±0.036)[c]

1j			0.099 (±0.022) [c]

[a]IC_{50} values were determined using a FRET-based assay against Lsp from *E. coli.* Mean (± SD) values are shown. [b][Lsp] = 400 nM. [c][Lsp] = 100 nM.

SAR of key analogues of **1a** are shown in Table 1 with comprehensive SAR lists shown in Tables S2-S4. Briefly, removal of NO_2 (**1b**) as well as restricting the conformation of R_3 with a cyclohexyl (**1c**) ablated the potency. Replacement of thiadiazole (**1d**) with oxadiazole led to an inactive compound (**1e**), indicating the importance of sulfur in the thiadiazole moiety. Elongation at R_1 from methyl to ethyl (**1f**) improved the potency to 1.2 μM. Further elongation (**1g, 1h, 1i**) as well as introduction of cyclopentyl moiety (**1j**) on R_1 leads to the improvement of the potency up to 0.1 μM. We observed a positive correlation between clogP (calculated using ChemDraw 16.0) and pIC_{50} in compound series that have different alkyl groups on R_1 position. This correlative relationship assisted in our design and selection for more potent inhibitors. Based on the inhibitory potency and relatively low lipophilicity (**1i**: clogP = 3.1, **1j**: clogP = 3.6), we chose compounds **1i** and **1j** for further biological characterization. Concentration-inhibition curves of compounds **1i** and **1j** are shown in Figure 3.

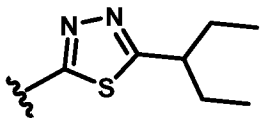
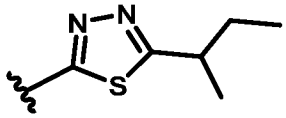
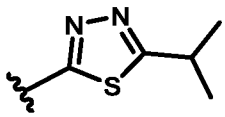
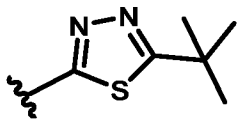
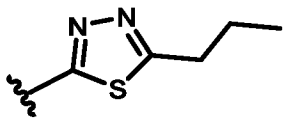
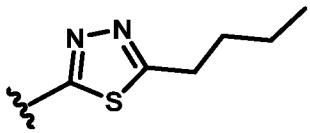
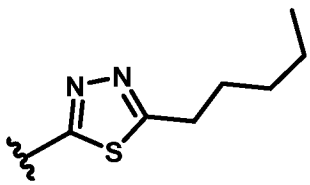
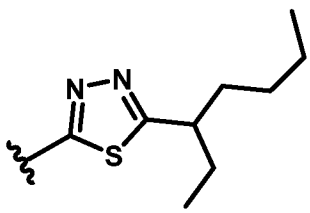
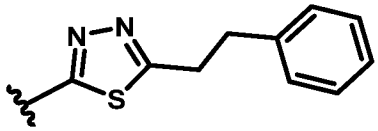
As a proof of concept, we tested the effects of these inhibitors on bacterial growth of *E. coli* (Figure 4). Although these compounds alone did not inhibit the growth of *E. coli*, Lsp inhibitors in combination with polymyxin B nonapeptide (PMBN), which improves the outer-membrane permeability of compounds,[25] slowed the growth of bacteria in a concentration-dependent manner (Figure 4A). Compound **1j** showed almost 100% growth inhibition at 25 μM (MIC = 25 μM = 11 μg/mL under this condition), while compound **1i** required a concentration of 100 μM to block growth (MIC = 100 μM = 41 μg/mL). Compound **1c**, which has IC_{50} > 400 μM in the enzyme assay (Table 1), did not inhibit the bacterial growth, suggesting that these effects are Lsp-inhibition dependent.

To further validate that the effects on the growth of *E. coli* are directly due to the inhibition of Lsp, we next measured the effects of **1j** on a diagnostic *E. coli* strain, YX23. YX23 is a mutant *E. coli* that has an IS*4* insertion in the gene that encodes Braun's lipoprotein, and was shown to be resistant to both myxovirescin and globomycin.[14] As shown in Figure 4B, compound **1j** inhibited the growth of the

isogenic parent strain (DW37) while it did not inhibit the growth of YX23. These data strongly suggest that the growth inhibitory effects of compound **1j** is specific toward Lsp.

Further data relating to structure-activity relationships are shown in Tables 2 - 7, and Figure 5, below.

Table 2

#	R_3	IC_{50} (μM)
Benzamide SR270728		4.2 ± 1.0
Sk061-69-2		8.7
Sk061-69-1		44
Sk061-69-3		36
Sk054-141-2		26
7960275		29 ± 0.60
Sk052-147-2		26
Sk061-69-4		2.5
Sk052-88-2		20

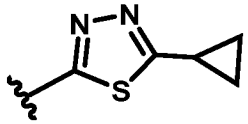
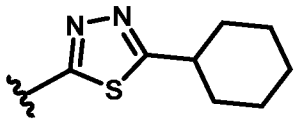
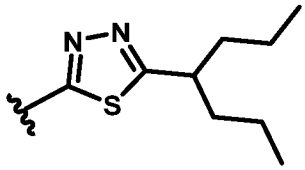
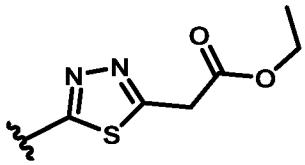
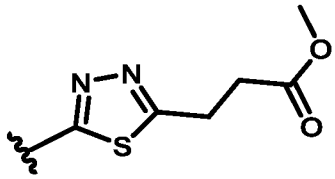
Sk052-147-1		208
Sk052-56		>400
Sk052-88-1		1.1
Sk052-27-2		78
Sk052-147-3		>200

Table 3

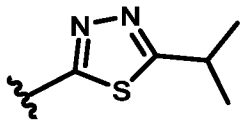
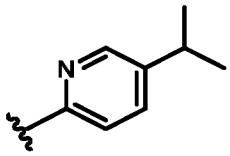
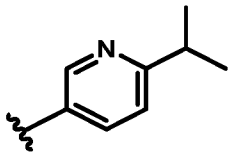
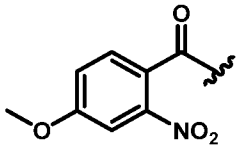
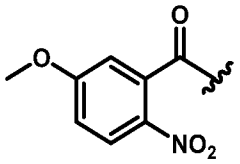
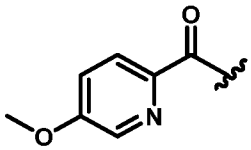
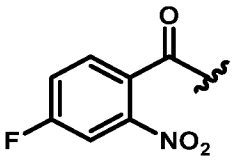
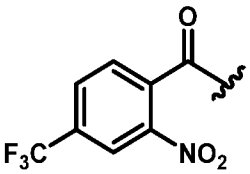
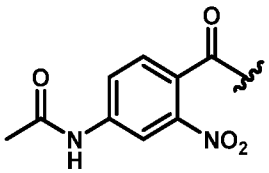
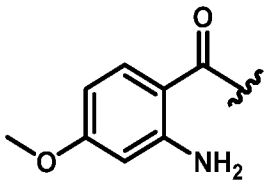
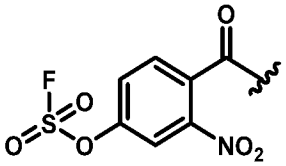
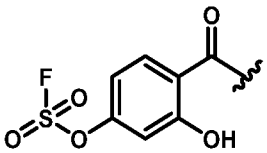
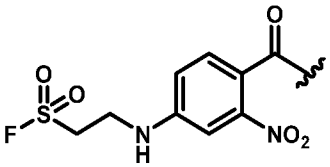
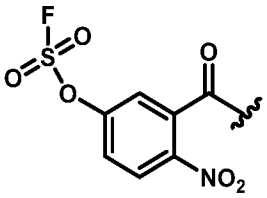
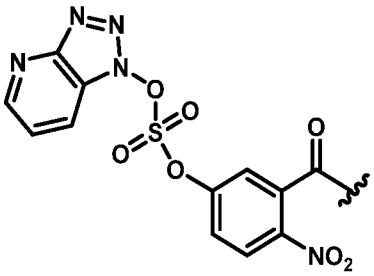
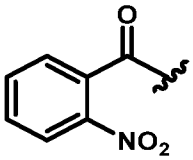
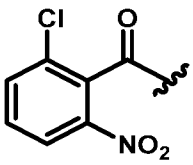
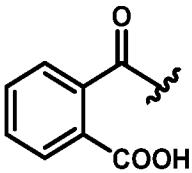
#	R	IC_{50} (μM)
Sk061-69-1		44
Sk056-82-3		>400
Sk056-82-4		>400

Table 4

#	R	IC_{50} (μM)
Sk052-146-2		2.2
Sk052-146-1		3.6
Sk054-91-2		>400
Sk054-91-3		7.2
Z123635394		9.3
Sk054-112-2		160
Sk054-142		11

Sk056-45-1		46
Sk056-50-4		290
Sk056-45-3		19
Sk054-144		65
Sk054-141-1		120
Sk054-91-1		11 ± 3
Sk056-82-2		260
Sk056-24		270

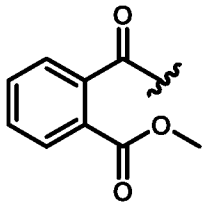
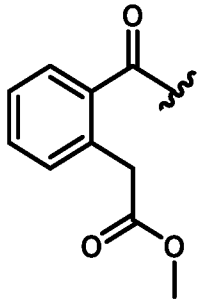
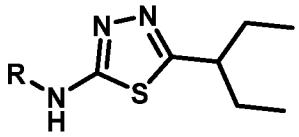
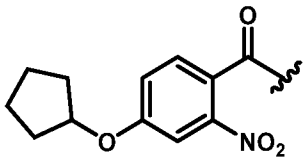
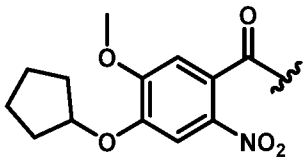
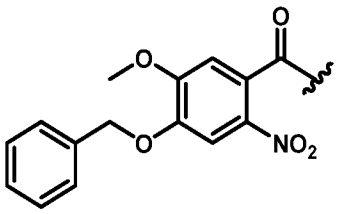
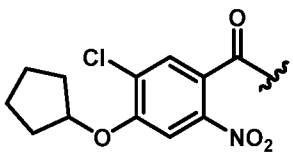
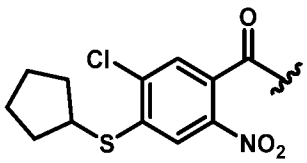
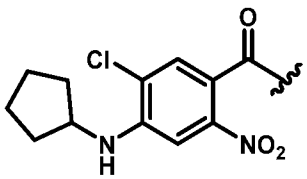
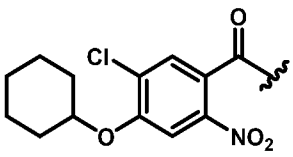
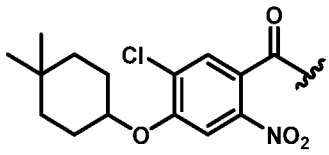
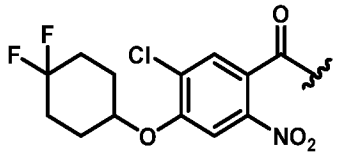
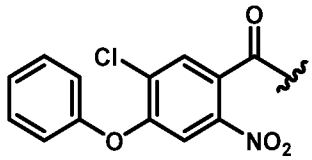
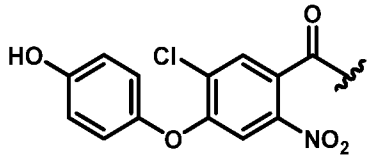
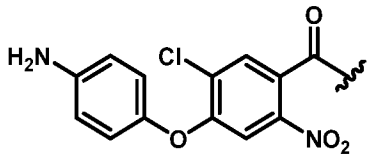
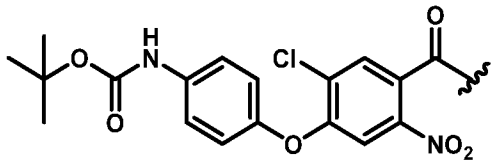
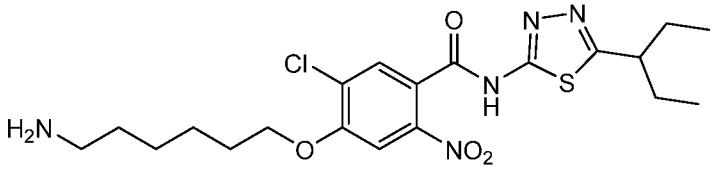
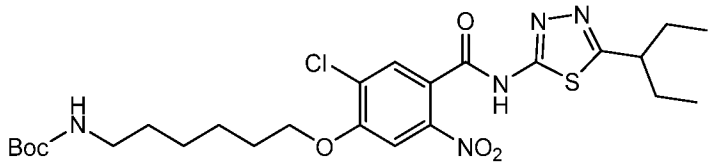
Sk056-13-2		12
Sk056-82-1		21

Table 5

#	R	IC_{50} (nM)
		
Sk054-116-1		400
Sk052-145-2		73
Sk054-82-3		2900
Sk054-116-2		110 ±12
Sk056-22-1		20
Sk056-16-1		170
Sk056-29		68

Sk061-31-1		230 E=100nM
Sk061-31-2		580 E=100nM
Sk056-22-2		190
Sk061-30-1 70%pure		>4,000 E=100nM
Sk061-32		~4,000 E=100nM
Sk061-29		640 E=100nM
Sk061-45		>4000 E=100nM
Sk061-54		~2300 E=100nM

[Lsp]=20 nM

Table 6

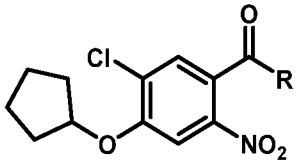
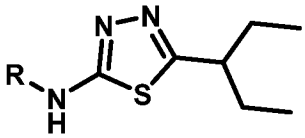
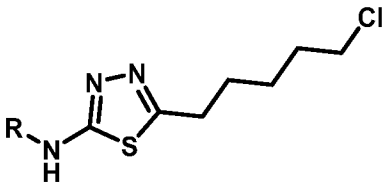
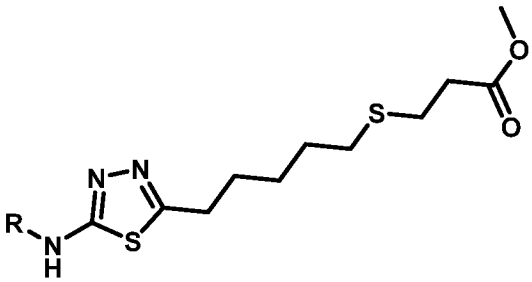
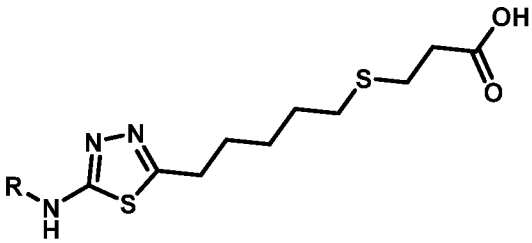
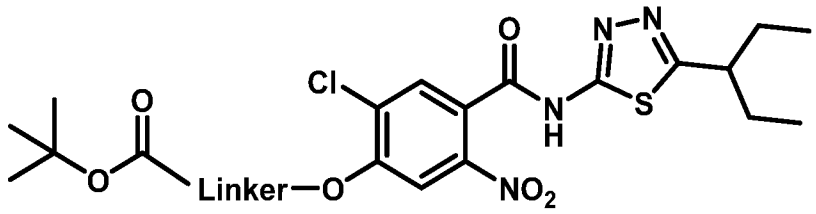
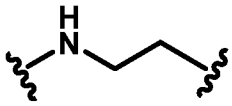
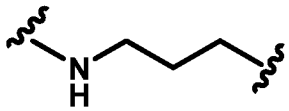
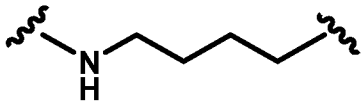
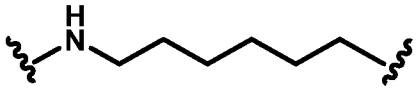
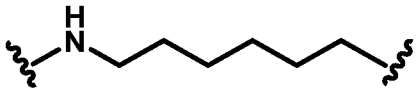
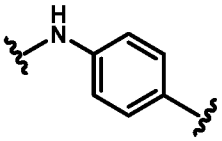
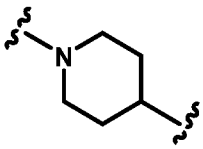
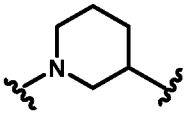
#	 R	IC_{50} (nM)
Sk054-116-2		110 ±12
Sk056-15		3,800
Sk056-26		3,200
Sk056-27		11,000

Table 7



#	Linker		IC_{50}[a] (μM) E=100nM
Sk061-68		2 (ethyl)	0.21 (0.53)
Sk061-80-1		3 (propyl)	1.7
Sk061-80-2		4 (butyl)	1.6
Sk061-80-3		5 (pentyl)	3.7
Sk061-54		6 (hexyl)	2.3
Sk061-29		p-Phe	0.64
Sk061-80-4		Piperidine	1.1
Sk061-80-5		piperidine	0.83

The effect of linker chain length from 2-6 carbon atoms (ethyl - hexyl) on IC50 is shown in Figure 5.

In summary, we have developed a high-throughput assay to monitor Lsp activity and have employed an HTS and medicinal chemistry campaign using this assay to look for Lsp inhibitors. Our study emphasizes the strength of combining chemical biology, HTS, and SAR for the development of chemical probes in microbiology and bacteriology as well as novel antibacterial candidates. The general SPPS method used to synthesize the Lsp FRET substrate can be rapidly optimized for lipid lengths and saturation, peptide sequence and length, and FRET pairs for any bacterial Lsp enzyme (Figure 2A). Importantly, we discovered novel small molecule *E. coli* Lsp inhibitors with our HTS assay. Structural optimization from a hit compound **1a** led to the generation of a class of molecules with nanomolar IC_{50} values *in vitro* as well as growth inhibitory properties against *E. coli*. These compounds will be ideal leads for further medicinal chemistry to improve the potency, permeability, solubility and selectivity, especially with our rapid assay and an x-ray structure of Lsp available.[8] Although high-throughput screening and target-based approach has been challenging for antibiotic development,[26,27] our work demonstrates the utility of HTS in the discovery of antimicrobial leads, as an alternative approach for traditional natural product-based approaches.[28,29] Our assay will stimulate the study of Lsp and bacterial lipoprotein maturation pathways, and our inhibitors further validate Lsp as an exciting new target for development of antibiotics.

Documents cited

[1] N. Buddelmeijer, *FEMS Microbiol. Rev.* **2015**, *39*, 246–261.

[2] M. Paetzel, A. Karla, N. Strynadka, *Chem. Rev.* **2002**, *102*, 4549–4580.

[3] M. Tokunaga, J. M. Loranger, H. C. Wu, *J. Biol. Chem.* **1984**, *259*, 3825–3830.

[4] M. M. Babu, M. L. Priya, A. T. Selvan, M. Madera, J. Gough, L. Aravind, K. Sankaran, *J. Bacteriol.* **2006**, *188*, 2761–2773.

[5] A. Kovacs-Simon, R. W. Titball, S. L. Michell, *Infect. Immun.* **2011**, *79*, 548–561.

[6] I. C. Sutcliffe, R. R. Russell, *J. Bacteriol.* **1995**, *177*, 1123–1128.

[7] M. T. N. Nguyen, T. Van Kersavond, S. H. L. Verhelst, *ACS Chem. Biol.* **2015**, *10*, 2423–2434.

[8] L. Vogeley, T. El Arnaout, J. Bailey, P. J. Stansfeld, C. Boland, M. Caffrey, *Science* **2016**, *351*, 876–880.

[9] M. Inukai, R. Enokita, A. Torikata, M. Nakahara, S. Iwado, M. Arai, *J. Antibiot.* **1978**, *31*, 410–420.

[10] M. Inukai, M. Takeuchi, K. Shimizu, M. Arai, *J. Antibiot.* **1978**, *31*, 1203–1205.

[11] K. Gerth, H. Irschik, H. Reichenbach, W. TROWITZSCH, *J. Antibiot.* **1982**, *35*, 1454–1459.

[12] H. Kogen, T. Kiho, M. Nakayama, *J. Am. Chem. Soc.* **2000**, *122*, 10214–10215.

[13] D. R. Williams, J. M. McGill, *J. Org. Chem.* **1990**, *55*, 3457–3459.

[14] Y. Xiao, K. Gerth, R. Müller, D. Wall, *Antimicrob. Agents Chemother.* **2012**, *56*, 2014–2021.

[15] T. Kiho, M. Nakayama, K. Yasuda, S. Miyakoshi, M. Inukai, H. Kogen, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2315–2318.

[16] Y. Paitan, E. Orr, E. Z. Ron, E. Rosenberg, *J. Bacteriol.* **1999**, *181*, 5644–5651.

[17] T. Kiho, H. Kogen, *J. Synth. Org. Chem.* **2005**, *1*, 51–62.

[18] Y. Xiao, D. Wall, *J. Bacteriol.* **2014**, *196*, 1174–1183.

[19] I. K. Dev, P. H. Ray, *J. Biol. Chem.* **1984**, *259*, 11114–11120.

[20] I. K. Dev, R. J. Harvey, P. H. Ray, *J. Biol. Chem.* **1985**, *260*, 5891–5894.

[21] M. Pouliot, S. Jeanmart, *J. Med. Chem.* **2016**, *59*, 497–503.

[22] J. B. Baell, G. A. Holloway, *J. Med. Chem.* **2010**, *53*, 2719–2740.

[23] J. L. Dahlin, J. W. M. Nissink, J. M. Strasser, S. Francis, L. Higgins, H. Zhou, Z. Zhang, M. A. Walters, *J. Med. Chem.* **2015**, *58*, 2091–2113.

[24] T. Kiho, M. Nakayama, K. Yasuda, S. Miyakoshi, M. Inukai, H. Kogen, *Bioorg. Med. Chem.* **2004**, *12*, 337–361.

[25] M. Vaara, *Microbiol. Rev.* **1992**, *56*, 395–411.

[26] D. J. Payne, M. N. Gwynn, D. J. Holmes, D. L. Pompliano, *Nat. Rev. Drug Discov.* **2007**, *6*, 29–40.

[27] R. Tommasi, D. G. Brown, G. K. Walkup, J. I. Manchester, A. A. Miller, *Nat. Rev. Drug Discov.* **2015**, *14*, 529–542.

[28] G. D. Wright, *Can. J. Microbiol.* **2014**, *60*, 147–154.

[29] C. T. Walsh, T. A. Wencewicz, *J. Antibiot.* **2014**, *67*, 7–22.

EXAMPLES

Experimental Section

General. All reagents and solvents were purchased from commercial suppliers and were used without further purification. Globomycin and polymyxin B nonapeptide hydrochloride, cationic cyclic peptide (PMBN) were purchased from Sigma-Aldrich (purity ≥ 98%). Compounds **1b** and **1d** were purchased from Enamine Ltd (purity ≥ 90%) or from ChemBridge, respectively. All reactions were performed in an inert atmosphere of dry nitrogen or argon. ^{1}H and ^{13}C NMR spectra were collected using a Bruker 600, 500, or 400 MHz spectrometer with chemical shifts reported relative to residual deuterated solvent peaks or a tetramethylsilane internal standard. Accurate masses were measured using an ESI-TOF (HRMS, Agilent MSD) or MSQ Plus mass spectrometer (LRMS, Thermo Scientific). Reactions were monitored on TLC plates (silica gel 60, F254 coating, EMD Millipore, 1057150001), and spots were either monitored under UV light (254 mm) or stained with phosphomolybdic acid. The same TLC system was used to test purity, and all final products showed a single spot on TLC with both phosphomolybdic acid and UV absorbance. The purity of the compounds that were tested in the assay was >95% based on ^{1}H NMR and reverse phase HPLC-UV on monitoring absorption at 254 nm. It should be noted that Lsp is susceptible to divalent cations such as Cu^{2+}, Zn^{2+}, thus care was taken to ensure that the final products did not contain contaminations of these metals.

Analytical LC method to determine the purity of synthetic compounds

Purity determination of synthetic compounds was performed on a Thermo Scientific Accela HPLC system using Accela 1250 pump. The Thermo Accucore C18 RP HPLC column (150 mm × 2.1 mm, particle size 2.6 µm) was used. The UV absorption between 190 nm and 400 nm was monitored, and the purity was determined by the peak area at 254 nm. Solvent used were Milli-Q water 99.9 / Formic acid 0.1, versus Acetonitrile 99.9 / Formic acid 0.1.

Expression and purification of Lsp from *E. coli*. The full-length Lsp clone from *E. coli* (residues 1-164) was generated using standard PCR-based cloning and verified via double-stranded plasmid sequencing. Lsp is over-expressed as a N-terminal His_6-tag fusion with an entrokinase cleavage site from *E. coli* Lemo21(DE3) (New England Biosciences) in a pET19b vector (Novagen). Cells were grown in 2xYT media supplemented with 50 µg/ml carbenicillin at 37 °C to an OD_{600} of 0.6-0.8.

Flasks were then transferred to 16 °C and protein expression was induced with 0.1 mM IPTG for 16 h. Cells were immediately harvested and resuspended in ice cold PBS, pH 7.4, 5% glycerol, 14 mM 2-mercaptoethanol (BME) (buffer A) supplemented with a Roche Complete inhibitor tablet (per 50 mL of buffer) and subjected to 3 cycles of lysis by microfluidization (Microfluidics). The cellular debris was removed by centrifugation at 24,000 x *g* for 15 min at 4 °C and the membrane fraction was isolated by subsequent ultracentrifugation at 100,000 x *g* for 1 hr at 4 °C. Membrane pellets was resuspended in buffer A containing 1% n-dodecyl-β-D-maltopyranoside (DDM) and stirred gently for 4 hr at 4 °C to solubilize the membrane. The membrane solutions were subjected to another round of ultracentrifugation at 100,000 x *g* for 45 min at 4 °C to remove non-soluble contents. A final concentration of 20 mM imidazole was added to the supernatant along with 5 ml of Ni-NTA bead slurry for 1 hr at 4 °C. Beads were washed 10 CVs of PBS, pH 7.4, 5% glycerol, 10 mM BME, 0.1% DDM (buffer B) and 50 mM imidazole and eluted with buffer B containing 250 mM imidazole. The eluted protein was concentrated down to 5 mL using Millipore Ultrafree-15 devices with a MWCO of 50,000 Da and subjected to gel filtration chromatography (Superdex 200, GE Amersham) in buffer B. Fractions containing Lsp were pooled, concentrated, and immediately stored at -70 °C. Pure Lsp yields are approximately 1-5 mg/L of culture with >95% purity, as assessed by SDS-PAGE.

Lsp FRET substrate synthesis. The Lsp peptide FRET substrate was designed based on known lipobox sequences and synthesized using standard Fmoc solid-phase synthesis chemistry starting with the coupling of Fmoc-Asp(EDANS)-OH (EMD Millipore) to TGR low-capacity resin (Novasyn). After completion of the peptide synthesis and the N-terminal capping with dabsyl chloride, the substrate was released from the resin with a cocktail of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (95%:2.5%:2.5%). Crude peptide was purified by normal-phase HPLC using an XBridge™ Prep Amide column (Waters, 5 µm, 19 x 100mm) with a 10-100% MeOH/DCM gradient as shown in the table below. The final purity of Lsp FRET substrate exceeded 95% purity on LC-UV (254 nm, figure below) and the structure was verified by HRMS (*m/z* Calcd. for ($M+H^+$) 1777.9705. Found 1777.9708).

Lsp activity assay, Michaelis-Menten kinetics, and IC_{50} determination. Michaelis-Menten kinetic analysis was performed with 400 nM of the wild type *E. coli* Lsp in a buffer consisting of PBS, pH 7.4, 5% glycerol, 0.5% DDM. To determine IC_{50}

values, Lsp was incubated at 10 - 400 nM in the presence of increasing amounts of inhibitor in a reaction buffer consisting of PBS, pH 7.4, 5% glycerol, 0.5% DDM, and 2.5% DMSO, and incubated for 30 min at 25 °C. 50 μM Lsp FRET substrate was subsequently added and the rate of substrate hydrolysis was measured by increase in fluorescence (excitation 355 nm, emission 495 nm) in 96-well plates on a PerkinElmer EnVision plate reader or Tecan Safire 2 microplate reader. Michaelis-Menten constants and IC_{50} values were determined using GraphPad Prism software (GraphPad, Inc.).

High-throughput screening. The HTS Lsp inhibitor assay protocol was modified based on optimized fluorescence that resulted from cleavage of Lsp FRET substrate by 200 nM *E. coli* Lsp in an activity buffer consisting of PBS, pH 7.4, 5% glycerol, 0.5% DDM, 6% DMSO and 10 mM DTT. The 96-well assay volume was miniaturized to 1536-well plates and performed in a total volume of 5 μL with 200 nM *E. coli* Lsp incubated with 8.4 μM compound (646,275 molecules total from the Scripps Drug Discovery Library) for 30 min at 25 °C prior to adding the FRET substrate to 20 μM. After a 60-min incubation, Lsp activity was quenched with 83 μM $ZnCl_2$ and the fluorescence was read on an EnVision plate reader (excitation 355 nm, emission 495 nm). The assay was well behaved, as evidenced by a Z' of 0.69 ± 0.05, signal-to-background (S:B) of 1.35 ± 0.05, hit cutoff of 27.9% inhibition, and 2271 inhibitors (0.35% hit rate). Globomycin and the DMSO vehicle served as the positive and negative controls, respectively, and all active compounds were re-screened in triplicate for verification as well as assessed for false-positive features against a counterscreen (*e.g.,* compounds added after quenching Lsp activity) with 344 molecules demonstrating selective Lsp activity (Z' = 0.74 ± 0.02, S:B = 1.41 ± 0.01). Subsequent 10-point titration curves identified 17 compounds with IC_{50} <5 μM with no response in the counterscreen conditions (Z' = 0.73 ± 0.03, S:B = 1.32 ± 0.01). Several molecules were eliminated from further study due to having known promiscuous reactive groups (*i.e.,* "PAINS"). Effect of DMSO concentration on Lsp activity is shown below ([Lsp] = 100 nM in 96 well plate format).

Bacteria growth assay. Effect of compounds on bacterial growth was measured using the method described previously with slight modifications[1]. Colony suspension method was used to prepare bacteria suspension using McFarland Standard 0.5 to adjust the turbidity. Cells were introduced to two-fold serial dilutions of compound in

a final volume of 105 µL with 5% DMSO. Mueller-Hinton broth (MH) or LB broth was used for *E. coli* ATCC25922 or *E. coli* DW37 and YX23, respectively. Plates were incubated at 37 °C, and the OD_{600} was measured using a PerkinElmer EnVision plate reader after gentle shaking. Mutant *E. coli* (DW37 and YX23) were previously described.[2] Globomycin was used as a positive control and the concentration-response curves of globomycin on bacteria growth (*E. coli* ATCC25922) is shown below (error bar: SD). See Figure 6.

***In vitro* stability measurements.**

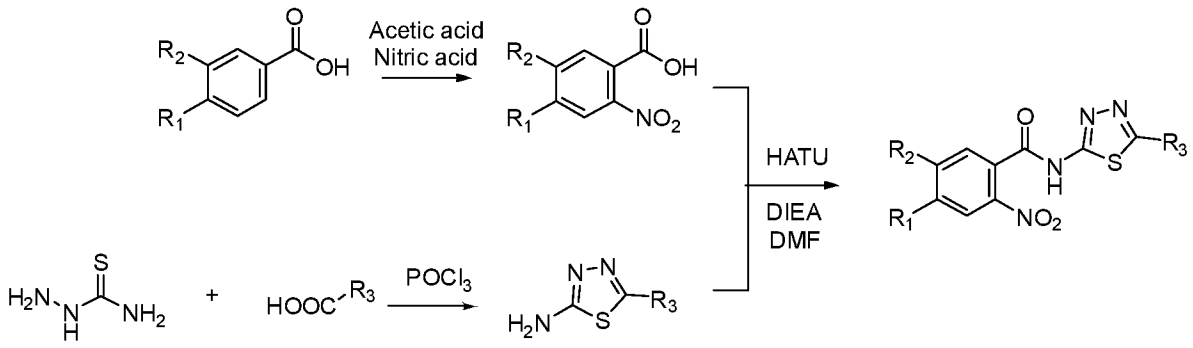
Microsomal stability:

Incubations were carried out in 96-well plates in 5 aliquots of 40 µL each (one for each time point) in duplicates. Liver microsomal incubation medium contained PBS (100 mM, pH 7.4), $MgCl_2$ (3.3 mM), NADPH (3 mM), glucose-6-phosphate (5.3 mM), glucose-6-phosphate dehydrogenase (0.67 units/mL) with 0.42 mg of human liver microsomal protein per mL. Control incubations were performed replacing the NADPH-cofactor system with PBS. Test compound (2 µM, final solvent concentration 1.6 %) was incubated with microsomes at 37 °C, shaking at 100 rpm. Five time points over 40 minutes had been analyzed. The reactions were stopped by adding 12 volumes of 90% acetonitrile-water to incubation aliquots, followed by protein sedimentation by centrifuging at 5500 rpm for 3 minutes. Supernatants were analyzed using the HPLC system coupled with tandem mass spectrometer using Shimadzu VP HPLC system including vacuum degasser, gradient pumps, reverse phase HPLC column, column oven and autosampler. The HPLC system was coupled with tandem mass spectrometer API 3000 (PE Sciex). The TurboIonSpray ion source was used in both positive and negative ion modes. Acquisition and analysis of the data were performed using Analyst 1.5.2 software (PE Sciex).

Plasma stability:

Incubations were carried out in 5 aliquots of 70 µL each (one for each time point), in duplicates. Test compounds (1 µM, final DMSO concentration 1%) were incubated at 37 °C with shaking at 100 rpm. Five time points over 120 minutes have been analyzed. The reactions were stopped by adding 420 µL of acetonitrile-water mixture (90:10) with subsequent plasma proteins sedimentation by centrifuging at 5500 rpm for 5 minutes. Supernatants were analyzed by the same HPLC system to the microsomal stability assay. The percentage of the test compounds remaining after incubation in plasma and their half-lives ($T_{1/2}$) were calculated.

Scheme 1: General synthetic scheme of benzamide compounds

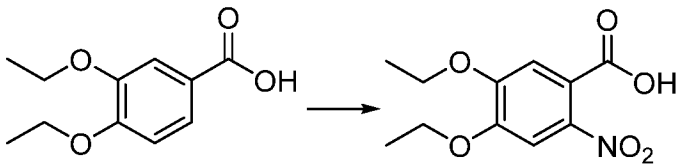


Synthesis of benzamide compounds.

Representative procedure for nitration of benzoic acid (Method A).

Nitrobenzoic acid was synthesized by the method described previously[3] with slight modifications.

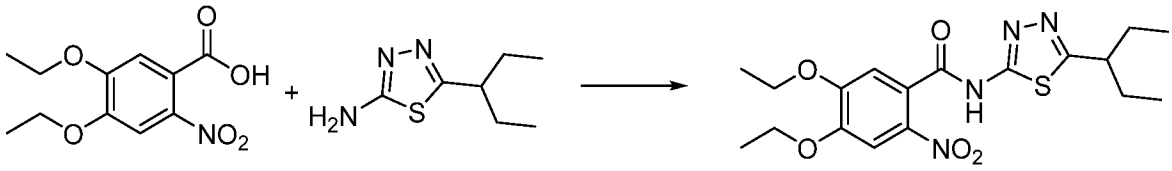
4,5-diethoxy-2-nitrobenzoic acid



A flask immersed in a room-temperature water bath was charged with 3,4-diethoxybenzoic acid (1.29 g. 6.1 mmol) and acetic acid (glacial, 5.2mL). HNO_3 (70%, 5.4 mL) was slowly added and stirred for 60 min at room temperature. The reaction was quenched upon addition of ice. A yellow precipitate formed that was filtered and washed with H_2O. Recrystallization from DCM gave *4,5-diethoxy-2-nitrobenzoic acid* as an off-white solid (424 mg, 1.7 mmol, 27%). ^1H NMR (600 MHz, $CDCl_3$) δ 10.29 (s, 1H), 7.37 (s, 1H), 7.24 (s, 1H), 4.24 - 4.17 (m, 4H), 1.51 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.6, 151.6, 150.9, 142.2, 119.0, 112.5, 108.1, 65.4, 65.4, 14.5, 14.4. HRMS (+) calcd for $(M+H)^+$ 256.0816. Found 256.0818.

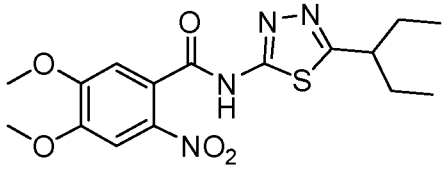
Representative procedure for amide coupling (Method B).

4,5-diethoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



To a DMF solution of 4,5-diethoxy-2-nitrobenzoic acid (75 mg, 299 µmol, 1.0 equiv) was added 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (598 µmol, 2.0 equiv), and stirred 10 min. To this solution was added 5-(pentan-3-yl)-1,3,4-thiadiazol-2-amine (104 mg, 598 µmol, 2.0 equiv), followed by diisopropylethylamine (0.5 mL), and stirred at RT overnight. To this solution was added saturated $NaHCO_3$ aqueous solution, and extracted with diethylether three times. The organic layer was combined, washed with saturated $NaHCO_3$ aqueous solution and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by flash column chromatography (DCM -> DCM:MeOH = 20:1) followed by recrystallization from DCM/acetone/hexane gave *4,5-diethoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide* as an off-white powder (88 mg, 215 µmol, 72%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.69 (s, 1H), 7.37 (s, 1H), 4.24 –4.18 (m, 4H), 2.99 (tt, *J* = 8.9, 5.3 Hz, 1H), 1.83 – 1.75 (m, 2H), 1.73 – 1.63 (m, 2H), 1.39-1.36 (m, 6H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.6, 164.4, 158.1, 152.3, 148.8, 139.0, 124.3, 112.2, 108.2, 65.0, 64.8, 43.9, 28.2, 14.39, 14.37, 11.6. HRMS (+) calcd for $(M+H)^+$ 409.1540. Found 409.1540. Purity (HPLC-UV): >99% (^{t}R= 9.7 min).

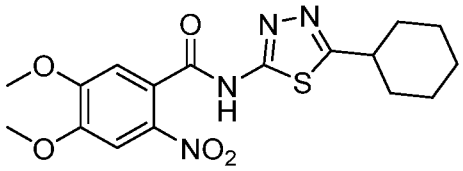
4,5-dimethoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide (**1a**)



The title compound was synthesized by the method B with slight modifications. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) was used instead of HATU, and DCM was used as a solvent both for reaction and extraction. Purification by flash column chromatography (DCM -> DCM:MeOH = 20:1) gave the title compound as a yellowish solid (56 mg, 25%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.04 (s, 1H), 7.72 (s, 1H), 7.39 (s, 1H), 3.93 (s, 3H), 3.92 (s, 3H), 3.00 (tt, *J* = 8.9, 5.3 Hz, 1H), 1.83 –1.76 (m, 2H), 1.74 – 1.63 (m, 2H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C

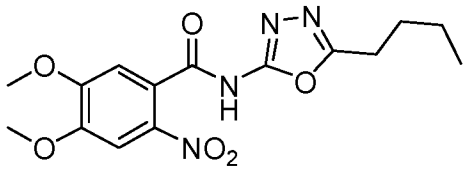
NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.8, 149.5, 139.0, 124.3, 111.4, 107.2, 56.6, 56.3, 43.7, 28.1, 11.5. LRMS (+) calcd for $(M+H)^+$ 381.1 Found 381.1. Purity (HPLC-UV): 95% (^{t}R= 8.4 min).

N-(5-cyclohexyl-1,3,4-thiadiazol-2-yl)-4,5-dimethoxy-2-nitrobenzamide (**1c**)



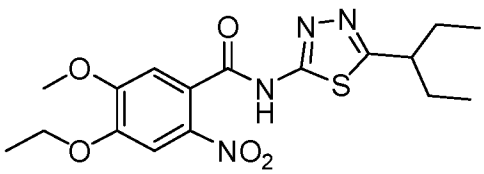
Method B (112 mg, off-white powder, 57%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.02 (s, 1H), 7.71 (s, 1H), 7.37 (s, 1H), 3.933 (s, 3H), 3.930 (s, 3H), 3.10 (tt, J = 11.3, 3.6 Hz, 1H), 2.10 – 2.03 (m, 2H), 1.80 –1.76 (m, 2H), 1.70 – 1.67 (m, 1H), 1.58 –1.51 (m, 2H), 1.46 – 1.37 (m, 2H), 1.29 – 1.26 (m, 1H). ^{13}C NMR (151 MHz, DMSO) δ 169.5, 164.3, 157.8, 152.8, 149.5, 139.0, 124.3, 111.4, 107.2, 56.6, 56.3, 38.6, 32.9, 25.3, 25.2. HRMS (+) calcd for $(M+H)^+$ 393.1227. Found 393.1228. Purity (HPLC-UV): 99% (^{t}R= 8.8 min).

N-(5-butyl-1,3,4-oxadiazol-2-yl)-4,5-dimethoxy-2-nitrobenzamide (**1e**)



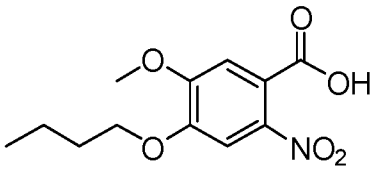
Method B (10 mg, brownish powder, 6 %).^{1}H NMR (600 MHz, DMSO-d_6) δ 7.93 (s, 1H), 7.75 (s, 1H), 7.42 (s, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 2.50 (t, J = 7.4 Hz, 2H), 1.50 – 1.43 (m, 2H), 1.30 – 1.23 (m, 2H), 0.84 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO) δ 164.5, 157.8, 153.6, 150.4, 149.7, 138.3, 122.5, 111.2, 106.8, 56.7, 56.3, 26.4, 24.3, 21.0, 13.2. LRMS (+) calcd for $(M+H)^+$ 351.1. Found 351.2. Purity (HPLC-UV): 95% (^{t}R= 8.1 min).

4-ethoxy-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide (**1f**)



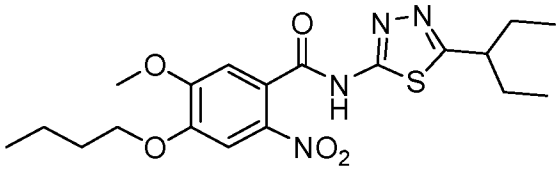
Method B (90 mg, off-white powder, 46 %).^{1}H NMR (600 MHz, DMSO-d_6) δ 13.04 (s, 1H), 7.69 (s, 1H), 7.39 (s, 1H), 4.20 (q, J = 6.9 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, J = 9.0, 5.4 Hz, 1H), 1.84 – 1.74 (m, 2H), 1.73 – 1.63 (m, 2H), 1.38 (t, J = 7.0 Hz, 3H), 0.85 (t, J = 7.4 Hz, 6H).^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.3, 157.9, 152.9, 148.7, 139.0, 124.1, 111.5, 107.9, 64.7, 56.6, 43.7, 28.1, 14.3, 11.5. LRMS (+) calcd for $(M+H)^+$ 395.1. Found 395.1. Purity (HPLC-UV): >99% (^{t}R= 9.5 min).

4-butoxy-5-methoxy-2-nitrobenzoic acid



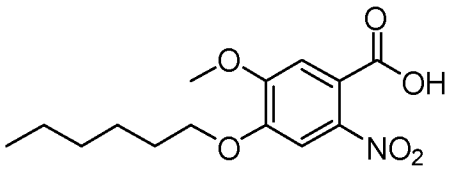
Method A (55 mg, off-white powder, 55 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.24 (s, 1H), 4.11 (t, J = 6.9 Hz, 2H), 3.98 (s, 3H), 1.90 –1.86 (m, 2H), 1.56-1.48 (m, 2H), 1.00 (t, J = 7.3 Hz, 3H). LRMS (-) calcd for $(M-H)^-$ 268.1. Found 268.2.

4-butoxy-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide (**1g**)



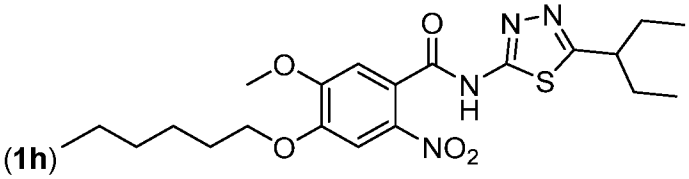
Method B (35 mg, off-white powder, 41 %).^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.70 (s, 1H), 7.38 (s, 1H), 4.14 (t, J = 6.5 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, J = 8.9, 5.4 Hz, 1H), 1.84 – 1.70 (m, 4H), 1.72 – 1.62 (m, 2H), 1.50 – 1.40 (m, 2H), 0.95 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.6, 164.4, 158.0, 153.0, 149.0, 139.2, 124.2, 111.6, 108.1, 68.8, 56.7, 43.9, 30.4, 28.2, 18.7, 13.7, 11.6. LRMS (+) calcd for $(M+H)^+$ 423.2. Found 423.1. Purity (HPLC-UV): 97% (^{t}R= 10.7 min).

4-(hexyloxy)-5-methoxy-2-nitrobenzoic acid



Method A (87 mg, off-white powder, 74 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.21 (s, 1H), 4.10 (t, *J* = 6.5 Hz, 2H), 3.98 (s, 3H), 1.91 – 1.85 (m, 2H), 1.48 – 1.44 (m, 2H), 1.37 – 1.34 (m, 4H), 0.92 (d, *J* = 6.6 Hz, 3H). LRMS (-) calcd for $(M-H)^-$ 296.1. Found 296.1.

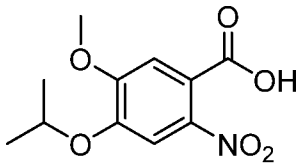
4-(hexyloxy)-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



(**1h**)

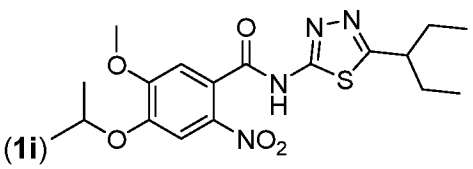
Method B (22 mg, off-white powder, 24 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.70 (s, 1H), 7.38 (s, 1H), 4.13 (t, *J* = 6.6 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, *J* = 8.9, 5.4 Hz, 1H), 1.84 – 1.72 (m, 4H), 1.70 – 1.65 (m, 2H), 1.47 – 1.39 (m, 2H), 1.37 – 1.27 (m, 4H), 0.90 – 0.83 (m, 9H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.9, 148.8, 139.0, 124.1, 111.5, 108.0, 68.9, 56.6, 43.7, 39.8, 30.8, 28.1, 24.9, 21.9, 13.8, 11.5. HRMS (+) calcd for $(M+H)^+$ 451.2010. Found 451.2013. Purity (HPLC-UV): 96% (^{t}R= 12.2 min).

4-isopropoxy-5-methoxy-2-nitrobenzoic acid



Method A (155 mg, yellowish solid, 53 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.23 (s, 1H), 4.73 – 4.61 (m, 1H), 3.97 (s, 3H), 1.44 (d, *J* = 6.1 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.1, 152.8, 149.7, 142.4, 118.9, 111.8, 109.4, 72.4, 56.6, 21.7.

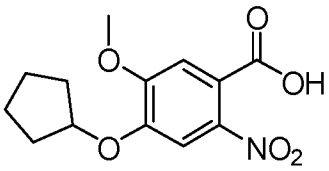
4-isopropoxy-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



(**1i**)

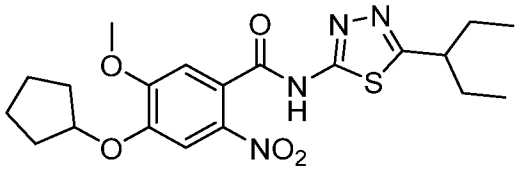
Method B (31 mg, yellowish powder, 18 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.01 (s, 1H), 7.70 (s, 1H), 7.38 (s, 1H), 4.81 (hept, J = 6.0 Hz, 1H), 3.92 (s, 3H), 2.99 (tt, J = 8.9, 5.4 Hz, 1H), 1.83 – 1.76 (m, 2H), 1.72 – 1.64 (m, 2H), 1.32 (d, J = 6.0 Hz, 6H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.1, 157.9, 153.6, 147.5, 139.1, 124.0, 111.8, 109.5, 71.3, 56.5, 43.7, 28.1, 21.4, 11.5. HRMS (+) calcd for $(M+H)^+$ 409.1540. Found 409.1541. Purity (HPLC-UV): 98% (^{t}R= 9.6 min).

4-(cyclopentyloxy)-5-methoxy-2-nitrobenzoic acid



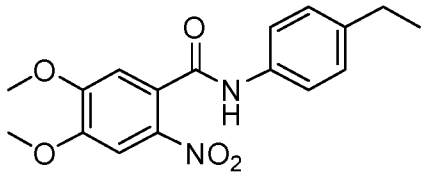
Method A (87 mg, yellowish powder, 73 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 (s, 1H), 7.22 (d, J = 2.8 Hz, 1H), 4.87 – 4.84 (m, 1H), 3.96 (s, 3H), 2.04 – 1.97 (m, 2H), 1.95 – 1.88 (m, 2H), 1.88 – 1.85 (m, 2H), 1.71 – 1.61 (m, 2H). LRMS (-) calcd for $(M-H)^-$ 280.1. Found 280.0.

4-(cyclopentyloxy)-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide (**1j**)



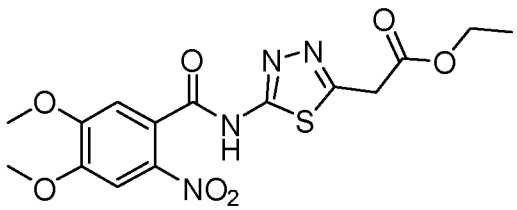
Method B (19 mg, off-white powder, 22 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.01 (s, 1H), 7.66 (s, 1H), 7.37 (s, 1H), 5.03 – 5.00 (m, 1H), 3.91 (s, 3H), 2.99 (tt, J = 9.3, 5.3 Hz, 1H), 2.00 – 1.95 (m, 2H), 1.83 – 1.66 (m, 8H), 1.66 – 1.58 (m, 2H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 158.0, 153.4, 147.7, 139.0, 123.9, 111.6, 109.1, 80.5, 56.6, 43.7, 31.9, 28.1, 23.5, 11.5. HRMS (+) calcd for $(M+H)^+$ 435.1697. Found 435.1697. Purity (HPLC-UV): 98% (^{t}R= 10.7 min).

N-(4-ethylphenyl)-4,5-dimethoxy-2-nitrobenzamide



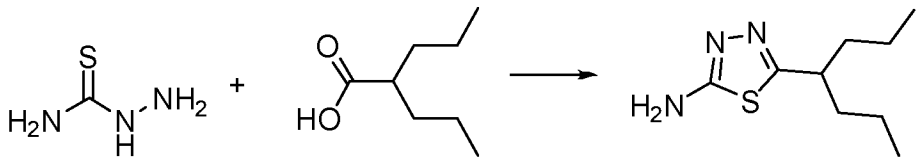
Method B (233 mg, off-white crystals, 71 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.41 (s, 1H), 7.69 (s, 1H), 7.58 – 7.55 (m, 2H), 7.25 (s, 1H), 7.20 – 7.17 (m, 2H), 3.94 (s, 3H), 3.92 (s, 3H), 2.58 (q, J = 7.6 Hz, 2H), 1.17 (t, J = 7.6 Hz, 3H). ^{13}C NMR (151 MHz, DMSO) δ 163.8, 152.9, 148.8, 139.1, 138.6, 136.6, 127.8, 127.3, 119.5, 110.9, 107.1, 56.5, 56.2, 27.6, 15.7. LRMS (+) calcd for $(M+H)^+$ 331.1. Found 331.2. Purity (HPLC-UV): >99% (^{t}R= 8.6 min).

Ethyl 2-(5-(4,5-dimethoxy-2-nitrobenzamido)-1,3,4-thiadiazol-2-yl)acetate



Method B (181 mg, yellowish crystals, 46 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.10 (s, 1H), 7.73 (s, 1H), 7.40 (s, 1H), 4.26 (s, 2H), 4.18 (q, J = 7.1 Hz, 2H), 3.94 (s, 6H), 1.24 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, DMSO) δ 168.9, 164.5, 159.7, 157.1, 153.0, 149.7, 139.1, 124.3, 111.5, 107.4, 61.2, 56.8, 56.4, 35.0, 14.1. LRMS (+) calcd for $(M+H)^+$ 397.1. Found 397.0. Purity (HPLC-UV): 98% (^{t}R= 6.7 min).

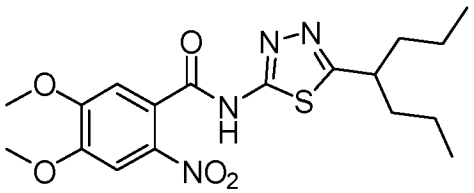
5-(heptan-4-yl)-1,3,4-thiadiazol-2-amine



The title compound was synthesized by the method described previously with slight modifications[4]. To an ice-cooled mixture of thiosemicarbazide (0.450 g, 4.95 mmol) and 2-propylpentanoic acid (0.713 g, 4.95 mmol), an excess of phosphorus oxychloride (0.9 mL, 9.9 mmol) was added slowly. Subsequently, the temperature was raised gradually to 75 °C. The reaction was kept at this temperature and stirred for 1 h. After cooling to RT, ice-water was added, and the mixture was stirred for an

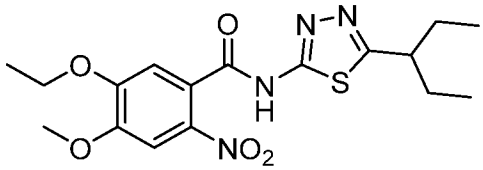
additional hour. The solution was extracted with ethyl acetate. The organic layer was combined, washed with aqueous saturated $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the title compound as an off-white solid (340 mg, 35 %). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.71 (s, 2H), 3.00 (tt, J = 9.3, 5.3 Hz, 1H), 1.65 – 1.50 (m, 4H), 1.28 -1.18 (m, 4H), 0.86 (t, J = 7.2 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.8, 162.8, 40.2, 36.5, 19.5, 13.6. LRMS (+) calcd for $(M+H)^+$ 200.1. Found 200.1.

N-(5-(heptan-4-yl)-1,3,4-thiadiazol-2-yl)-4,5-dimethoxy-2-nitrobenzamide



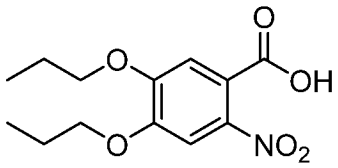
Method B (95 mg, off-white powder, 47%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.05 (s, 1H), 7.72 (s, 1H), 7.39 (s, 1H), 3.94 (s, 3H), 3.93 (s, 3H), 3.17 (tt, J = 9.3, 5.3 Hz, 1H), 1.75 – 1.61 (m, 4H), 1.29 – 1.18 (m, 4H), 0.88 (t, J = 7.3 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.9, 164.2, 157.9, 152.8, 149.5, 139.0, 124.3, 111.4, 107.2, 56.6, 56.3, 37.8, 30.9, 19.8, 13.6. LRMS (+) calcd for $(M+H)^+$ 409.2. Found 409.1. Purity (HPLC-UV): 99% (^{t}R= 10.6 min).

5-ethoxy-4-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



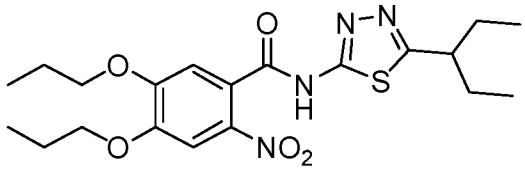
Method B (46 mg, off-white powder, 23 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.71 (s, 1H), 7.37 (s, 1H), 4.21 (q, J = 7.0 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, J = 8.9, 5.3 Hz, 1H), 1.81 – 1.77 (m, 2H), 1.70 – 1.67 (m, 2H), 1.37 (t, J = 7.0 Hz, 3H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.1, 149.5, 138.8, 124.3, 111.9, 107.3, 64.9, 56.2, 43.7, 28.1, 14.3, 11.5. LRMS (+) calcd for $(M+H)^+$ 395.1. Found 395.1. Purity (HPLC-UV): >99% (^{t}R= 9.6 min).

2-nitro-4,5-dipropoxybenzoic acid



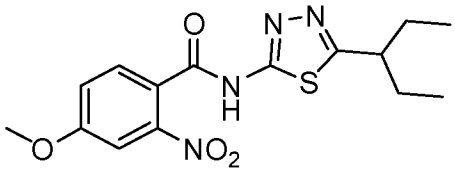
Method A (424 mg, yellowish solid, 27 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.22 (s, 1H), 4.10 – 4.04 (m, 4H), 1.96 – 1.85 (m, 4H), 1.10 – 1.04 (m, 6H).

2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)-4,5-dipropoxybenzamide



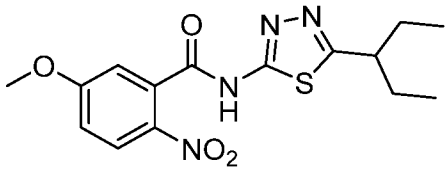
Method B (33 mg, off-white powder, 33 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.01 (s, 1H), 7.69 (s, 1H), 7.38 (s, 1H), 4.13 – 4.10 (m, 4H), 3.03 – 2.95 (m, 1H), 1.83 – 1.73 (m, 6H), 1.72 – 1.64 (m, 2H), 1.01 – 0.98 (m, 6H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.5, 148.9, 138.8, 124.2, 112.3, 108.4, 70.5, 70.3, 43.7, 28.1, 21.7, 21.6, 11.5, 10.1, 10.1. LRMS (+) calcd for $(M+H)^+$ 437.2. Found 436.9. Purity (HPLC-UV): 98% (^{t}R= 11.5 min).

4-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



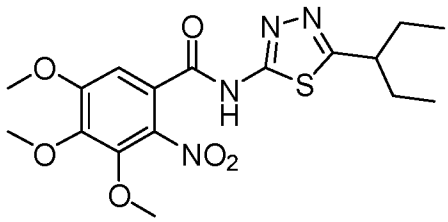
Method B (49 mg, white powder, 35 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.15 (s, 1H), 7.82 (d, *J* = 8.6 Hz, 1H), 7.65 (d, *J* = 2.6 Hz, 1H), 7.42 (dd, *J* = 8.6, 2.6 Hz, 1H), 3.93 (s, 3H), 2.98 (ddd, *J* = 10.6, 9.1, 5.3 Hz, 1H), 1.78 (dtd, *J* = 14.7, 7.4, 5.4 Hz, 2H), 1.73 – 1.62 (m, 2H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.6, 164.0, 161.3, 158.1, 148.7, 131.3, 121.5, 118.8, 109.8, 56.4, 43.9, 28.1, 11.6. LRMS (+) calcd for $(M+H)^+$ 351.1. Found 351.1. Purity (HPLC-UV): 99% (^{t}R= 8.6 min).

5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



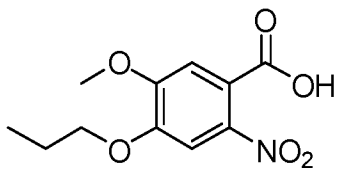
Method B (56 mg, white powder, 40 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.08 (s, 1H), 8.22 (d, *J* = 9.2 Hz, 1H), 7.36 (d, *J* = 2.8 Hz, 1H), 7.27 (dd, *J* = 9.2, 2.8 Hz, 1H), 3.93 (s, 3H), 3.01 – 2.98 (m, 1H), 1.82 – 1.79 (m, 2H), 1.74 – 1.63 (m, 2H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.7, 164.3, 163.6, 157.8, 138.6, 133.2, 127.1, 116.2, 114.7, 56.7, 43.8, 28.2, 11.6. LRMS (+) calcd for $(M+H)^+$ 351.1. Found 351.1. Purity (HPLC-UV): 99% (^{t}R= 8.5 min).

3,4,5-trimethoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



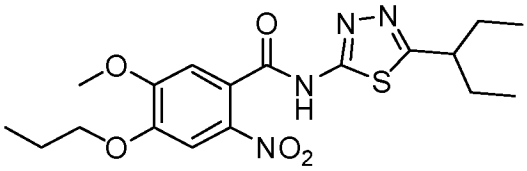
Method B (127 mg, off-white powder, 62 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.39 (broad s, 1H), 7.54 (s, 1H), 3.98 (s, 3H), 3.91 (s, 6H), 2.97 (tt, *J* = 8.9, 5.4 Hz, 1H), 1.77 (dtd, *J* = 14.8, 7.4, 5.4 Hz, 2H), 1.72 – 1.61 (m, 2H), 0.83 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.4, 162.2, 158.6, 154.1, 145.5, 144.5, 138.2, 121.5, 108.4, 62.5, 60.9, 56.7, 43.8, 28.0, 11.4. LRMS (+) calcd for $(M+H)^+$ 411.1. Found 411.1. Purity (HPLC-UV): 98% (^{t}R= 10.1 min).

5-methoxy-2-nitro-4-propoxybenzoic acid



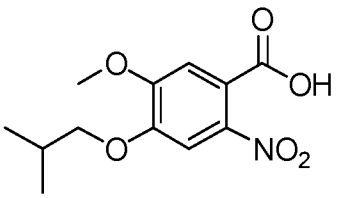
Method A (52 mg, off-white powder, 43 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.23 (s, 1H), 4.07 (t, *J* = 6.8 Hz, 2H), 3.98 (s, 3H), 1.92 (m, 2H), 1.07 (t, *J* = 7.3 Hz, 3H). LRMS (-) calcd for $(M-H)^-$ 254.1. Found 254.0.

5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)-4-propoxybenzamide



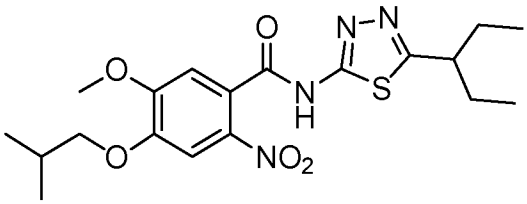
Method B (25 mg, off-white powder, 31%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.69 (s, 1H), 7.39 (s, 1H), 4.10 (t, J = 6.6 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, J = 8.9, 5.4 Hz, 1H), 1.84 – 1.74 (m, 4H), 1.71 – 1.64 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.9, 148.8, 139.0, 124.1, 111.5, 108.0, 70.4, 56.6, 43.7, 28.1, 21.7, 11.5, 10.2. LRMS (+) calcd for $(M+H)^+$ 409.2. Found 409.0. Purity (HPLC-UV): 98% (^{t}R= 10.5 min).

4-isobutoxy-5-methoxy-2-nitrobenzoic acid



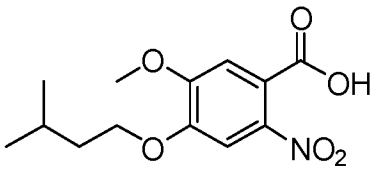
Method A (56 mg, off-white powder, 47 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 (d, J = 2.8 Hz, 1H), 7.23 (d, J = 2.7 Hz, 1H), 3.98 (s 3H), 3.85 (d, J = 6.7 Hz, 2H), 2.24 – 2.15 (m, 1H), 1.06 (d, J = 6.7 Hz, 6H). LRMS (-) calcd for $(M-H)^-$ 268.1. Found 268.1.

4-isobutoxy-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



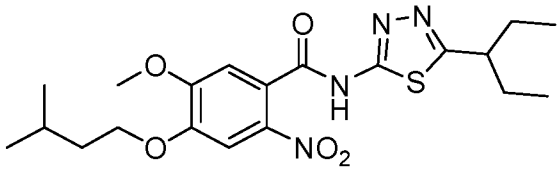
Method B (37 mg, yellowish crystals, 42 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.70 (s, 1H), 7.39 (s, 1H), 3.96 – 3.90 (m, 5H), 2.99 (tt, J = 8.9, 5.4 Hz, 1H), 2.11 – 2.04 (m, 1H), 1.83 – 1.75 (m, 2H), 1.72 – 1.64 (m, 2H), 1.00 (d, J = 6.7 Hz, 6H), 0.85 (t, J = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.6, 164.3, 158.0, 153.1, 149.0, 139.1, 124.2, 111.6, 108.1, 75.1, 56.8, 43.8, 28.2, 27.6, 18.9, 11.6. LRMS (+) calcd for $(M+H)^+$ 423.2. Found 423.1. Purity (HPLC-UV): 98% (^{t}R= 10.8 min).

4-(isopentyloxy)-5-methoxy-2-nitrobenzoic acid



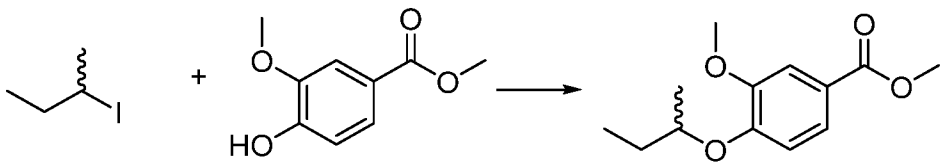
Method A (97 mg, off-white solid, 33 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.23 (s, 1H), 4.13 (t, *J* = 6.7 Hz, 2H), 3.98 (s, 3H), 1.88 – 1.79 (m, 1H), 1.79 – 1.76 (m, 2H), 0.99 (d, *J* = 6.5 Hz, 6H). LRMS (-) calcd for $(M-H)^-$ 282.1. Found 282.2.

4-(isopentyloxy)-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



Method B (20 mg, yellowish powder, 23 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.03 (s, 1H), 7.73 (s, 1H), 7.38 (s, 1H), 4.17 (t, *J* = 6.7 Hz, 2H), 3.93 (s, 3H), 2.99 (tt, *J* = 8.8, 5.4 Hz, 1H), 1.83 – 1.75 (m, 3H), 1.72 – 1.64 (m, 4H), 0.95 (d, *J* = 6.6 Hz, 6H), 0.85 (t, *J* = 7.4 Hz, 6H).^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 152.9, 148.8, 139.1, 124.1, 111.5, 108.0, 67.4, 56.6, 43.7, 37.0, 28.1, 24.4, 22.3, 11.5. LRMS (+) calcd for $(M+H)^+$ 437.2. Found 436.8. Purity (HPLC-UV): 96% (^{t}R= 11.3 min).

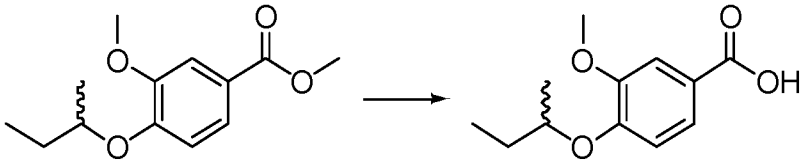
methyl 4-(sec-butoxy)-3-methoxybenzoate



The title compound was synthesized by the method described previously with slight modifications[5]. To a DMF solution of methyl 4-hydroxy-3-methoxybenzoate (440 mg, 2.4 mmol, 1 equiv) was slowly added sodium hydride (60 % dispersion in mineral oil, 150 mg, 3.75 mmol, 1.56 equiv) followed by 2-iodobutane (1.4 mL, 12 mmol, 5 equiv). The solution was heated to 65°C and stirred 4 h. After cooling to rt, aqueous saturated LiCl was added and extracted with ethyl acetate. The organic layer was combined and washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by flash column chromatography (Hexane: ethyl

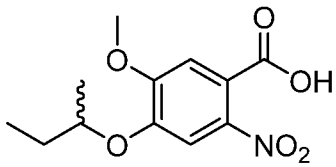
acetate = 5:1) gave the title compound as a white powder (200 mg, 35 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.55 (d, *J* = 2.0 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H), 4.42 – 4.35 (m, 1H), 3.90 – 3.88 (m, 6H), 1.89 – 1.77 (m, 1H), 1.74 – 1.60 (m, 1H), 1.35 (d, *J* = 6.1 Hz, 3H), 0.99 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 166.9, 151.9, 149.7, 123.4, 122.4, 113.4, 112.8, 76.5, 56.1, 51.9, 29.1, 19.2, 9.8.

4-(sec-butoxy)-3-methoxybenzoic acid



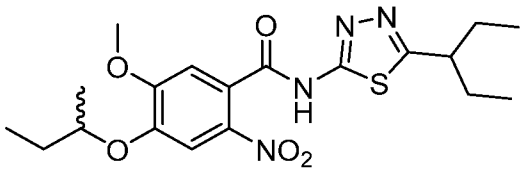
To a solution of methyl 4-(sec-butoxy)-3-methoxybenzoate (200 mg, 0.84 mmol) in methanol (20 mL) was added 2M NaOHaq solution (10 mL) and stirred for 1 hour. To the reaction mixture was added 1 M HCl aqueous solution (50 mL) and extracted with ethyl acetate three times. The organic layer was combined and washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the title compound as an off-white solid (180 mg, 96 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.13 (s, 1H), 7.75 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.62 (d, *J* = 2.0 Hz, 1H), 6.91 (d, *J* = 8.4 Hz, 1H), 4.44 – 4.38 (m, 1H), 3.92 (s, 3H), 1.90 – 1.81 (m, 1H), 1.72 – 1.63 (m, 1H), 1.37 (d, *J* = 6.1 Hz, 3H), 1.00 (t, *J* = 7.5 Hz, 3H).

4-(sec-butoxy)-5-methoxy-2-nitrobenzoic acid



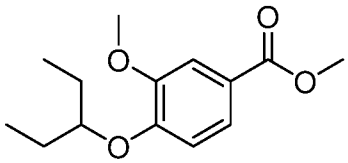
Method A (51 mg, off-white solid, 24 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.36 (s, 1H), 7.26 (s, 1H), 4.45 –4.40 (m, 1H), 3.98 (s, 3H), 1.90 – 1.82 (m, 1H), 1.74 – 1.70 (m, 1H), 1.39 (d, *J* = 6.1 Hz, 3H), 1.01 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.2, 152.8, 150.2, 142.5, 118.6, 111.9, 109.4, 77.6, 56.6, 28.9, 19.0, 9.7.

4-(sec-butoxy)-5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)benzamide



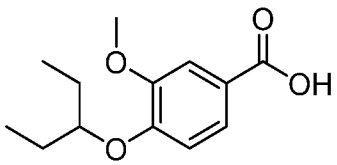
Method B (30 mg, yellowish powder, 37 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.01 (s, 1H), 7.70 (s, 1H), 7.38 (s, 1H), 4.64 – 4.59 (m, 1H), 3.93 (s, 3H), 2.99 (tt, *J* = 8.9, 5.3 Hz, 1H), 1.84 – 1.58 (m, 6H), 1.28 (d, *J* = 6.1 Hz, 3H), 0.94 (t, *J* = 7.4 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 153.6, 147.8, 139.1, 123.9, 111.8, 109.5, 76.1, 56.6, 43.7, 28.2, 28.1, 18.6, 11.5, 9.3. HRMS (+) calcd for $(M+H)^+$ 423.1697. Found 423.1698. Purity (HPLC-UV): >99% (^{t}R= 10.4 min).

methyl 3-methoxy-4-(pentan-3-yloxy)benzoate



The title compound was synthesized by the method described for *methyl 4-(sec-butoxy)-3-methoxybenzoate* using 3-bromopentane. It should be noted that a catalytic amount of NaI was added to the reaction mixture. (334 mg, off-white powder, 37 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (d, *J* = 8.2 Hz, 1H), 7.56 (d, *J* = 1.9 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 4.22 –4.18 (m, 1H), 3.90 (s, 3H), 3.88 (s, 3H), 1.79 – 1.66 (m, 4H), 0.97 (t, *J* = 7.5 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 166.7, 152.4, 149.7, 123.3, 122.2, 113.5, 112.8, 81.6, 55.9, 51.7, 26.1, 9.5.

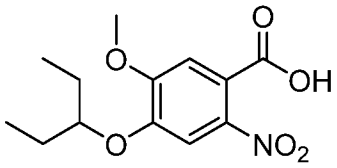
3-methoxy-4-(pentan-3-yloxy)benzoic acid



The title compound was synthesized by the method described for *4-(sec-butoxy)-3-methoxybenzoic acid* from *methyl 3-methoxy-4-(pentan-3-yloxy)benzoate.* (170 mg, off-white solid, 54 %).

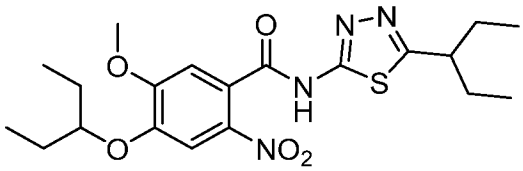
^{1}H NMR (600 MHz, $CDCl_3$) δ 11.81 (s, 1H), 7.74 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.62 (d, *J* = 2.0 Hz, 1H), 6.91 (d, *J* = 8.6 Hz, 1H), 4.26 – 4.21 (m, 1H), 3.91 (s, 3H), 1.80 – 1.65 (m, 4H), 0.98 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.2, 153.4, 149.7, 124.5, 121.4, 113.4, 113.3, 81.9, 56.1, 26.3, 9.7.

5-methoxy-2-nitro-4-(pentan-3-yloxy)benzoic acid



Method A (29 mg, off-white powder, 14 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 (s, 1H), 7.26 (s, 1H), 4.25 (p, *J* = 5.9 Hz, 1H), 3.98 (s, 3H), 1.76 (qdd, *J* = 7.3, 5.9, 3.9 Hz, 4H), 0.98 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.3, 152.9, 150.7, 142.6, 118.5, 112.0, 109.6, 82.9, 56.6, 26.0, 9.6.

5-methoxy-2-nitro-N-(5-(pentan-3-yl)-1,3,4-thiadiazol-2-yl)-4-(pentan-3-yloxy)benzamide



Method B (20 mg, off-white powder, 45 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.00 (s, 1H), 7.71 (s, 1H), 7.39 (s, 1H), 4.48 (p, *J* = 5.8 Hz, 1H), 3.93 (s, 3H), 2.99 (tt, *J* = 8.9, 5.3 Hz, 1H), 1.79 (dtd, *J* = 14.8, 7.3, 5.3 Hz, 2H), 1.73 – 1.61 (m, 6H), 0.91 (t, *J* = 7.4 Hz, 6H), 0.85 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 168.5, 164.2, 157.9, 153.6, 148.3, 139.1, 123.8, 111.9, 109.5, 80.9, 56.6, 43.7, 28.1, 25.2, 11.5, 9.1. HRMS (+) calcd for $(M+H)^+$ 437.1853. Found 437.1855. Purity (HPLC-UV): >99% (^{t}R= 11.1 min).

<u>Documents Cited in Examples Section</u>

[1] M. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical, D. Infectious, *Clin. Microbiol. Infect.* **2003**, *9*, ix-xv; b) I. Wiegand, K. Hilpert, R. E. W. Hancock, *Nat. Protocols* **2008**, *3*, 163-175.

[2] Y. Xiao, K. Gerth, R. Müller, D. Wall, *Antimicrob. Agents Chemother.* **2012**, *56*, 2014-2021.

[3] H. F. VanBrocklin, J. K. Lim, S. L. Coffing, D. L. Hom, K. Negash, M. Y. Ono, J. L. Gilmore, I. Bryant, D. J. Riese, *J. Med. Chem.* **2005**, *48*, 7445-7456.

[4] S. Ferrari, F. Morandi, D. Motiejunas, E. Nerini, S. Henrich, R. Luciani, A. Venturelli, S. Lazzari, S. Calò, S. Gupta, V. Hannaert, P. A. M. Michels, R. C. Wade, M. P. Costi, *J. Med. Chem.* **2011**, *54*, 211-221.

[5] G. Wang, L. Beigelman, A. Truong, C. Napolitano, D. Andreotti, H. He, K. Stein, Ann;, *PCT/US2014/051642* **2015**.

While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements will be apparent to those skilled in the art without departing from the spirit and scope of the claims.

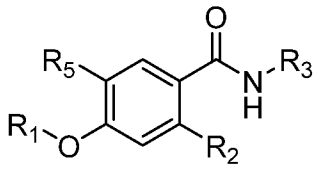
All patents and publications referred to herein are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A compound of formula (1)



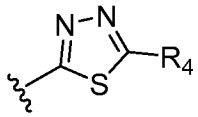
(1),

wherein

R_1 is (C1-C6) alkyl, or is a 5-, 6- or 7-membered cycloalkyl;

R_2 is H, NO_2, halo, or trifluoromethyl;

R_3 is a 1,3,4-thiadiazole of formula



,

wherein R_4 is (C4-C6) straight or branched chain alkyl, or is a 5-, 6- or 7-membered cycloalkyl;

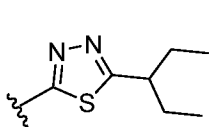
R_5 is H or (C1-C4)alkoxyl;

or a pharmaceutically acceptable salt thereof.

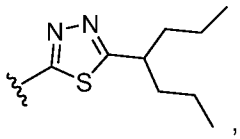
2. The compound of claim 1, wherein R_1 is isopropyl or cyclopentyl.

3. The compound of claim 1, wherein R_2 is NO_2.

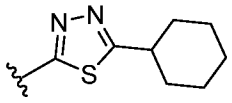
4. The compound of claim 1, wherein R_3 is any one of



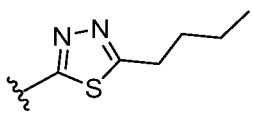
,



,



, or



.

5. A method of inhibiting a bacterial lipoprotein signal peptidase (Lsp), comprising contacting the peptidase with an effective amount or concentration of a compound of claim 1.

6. A method of treatment of a bacterial infection in a patient, comprising administering to the patient an effective dose of a compound of claim 1.

7. A method of screening compounds for inhibitory bioactivity of a bacterial lipoprotein signal peptidase (Lsp), comprising contacting a Lsp peptide FRET substrate, comprising a hexapeptide VTGCAK, with a N-terminal dabsyl quencher and C-terminal EDANS fluorophore, wherein the cysteine residue of the hexapeptide is S-alkylated with a diacylglycerol residue, and a candidate inhibitor compound, then measuring fluorescence from the fluorophore signalling cleavage of the Lsp FRET substrate and its inhibition by the candidate inhibitor.

8. The method of claim 7 wherein screening of multiple compounds are carried out in parallel in a High Throughput Screening format.

FIGURES

Figure 1

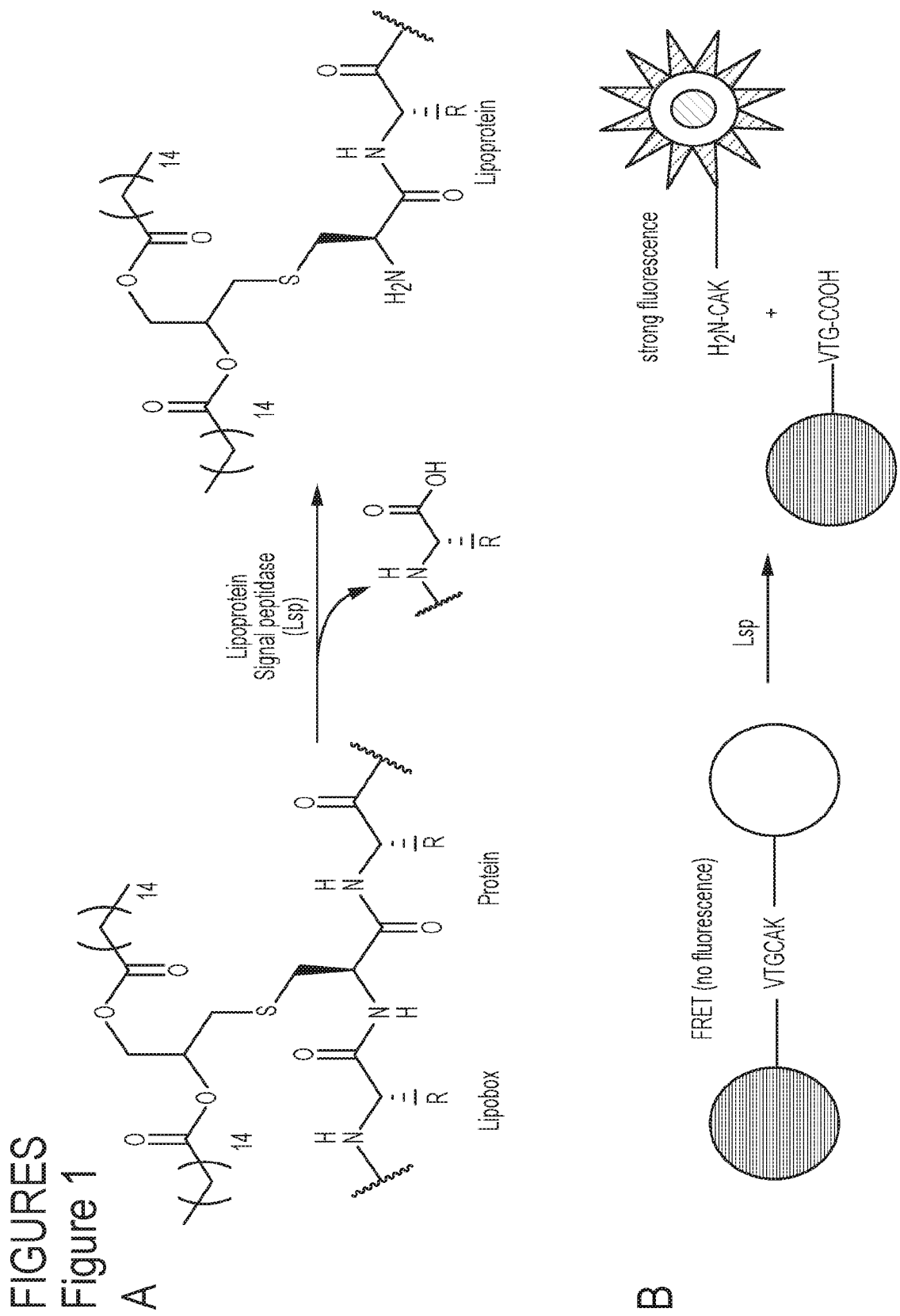
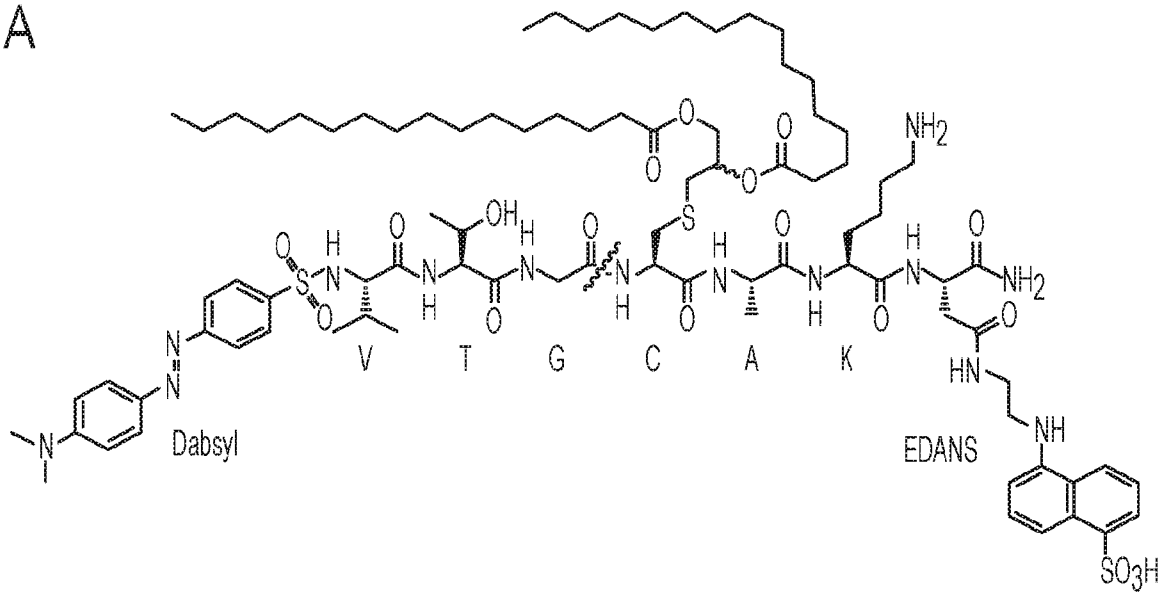


Figure 2

A


NH2
OH
S
V T G C A K
HN
NH
Dabsyl
EDANS
SO3H

B

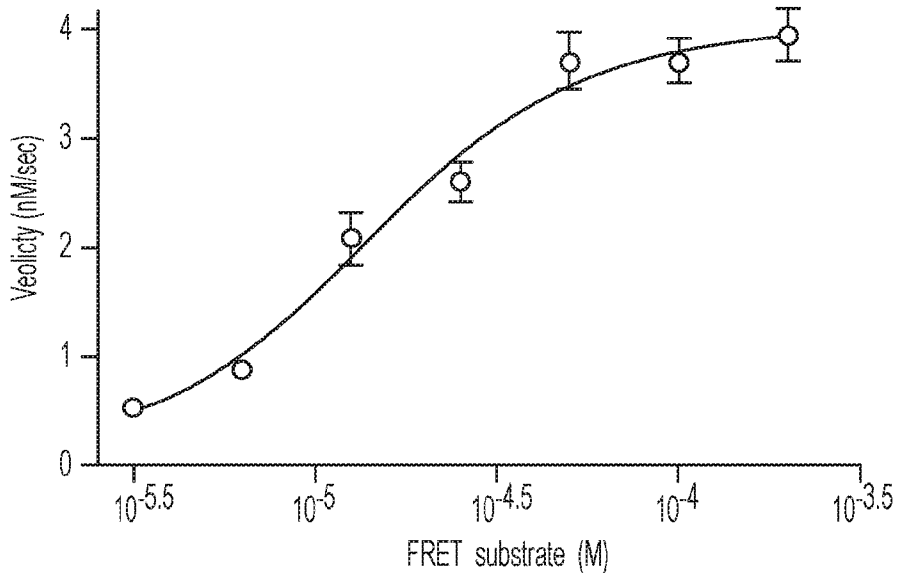
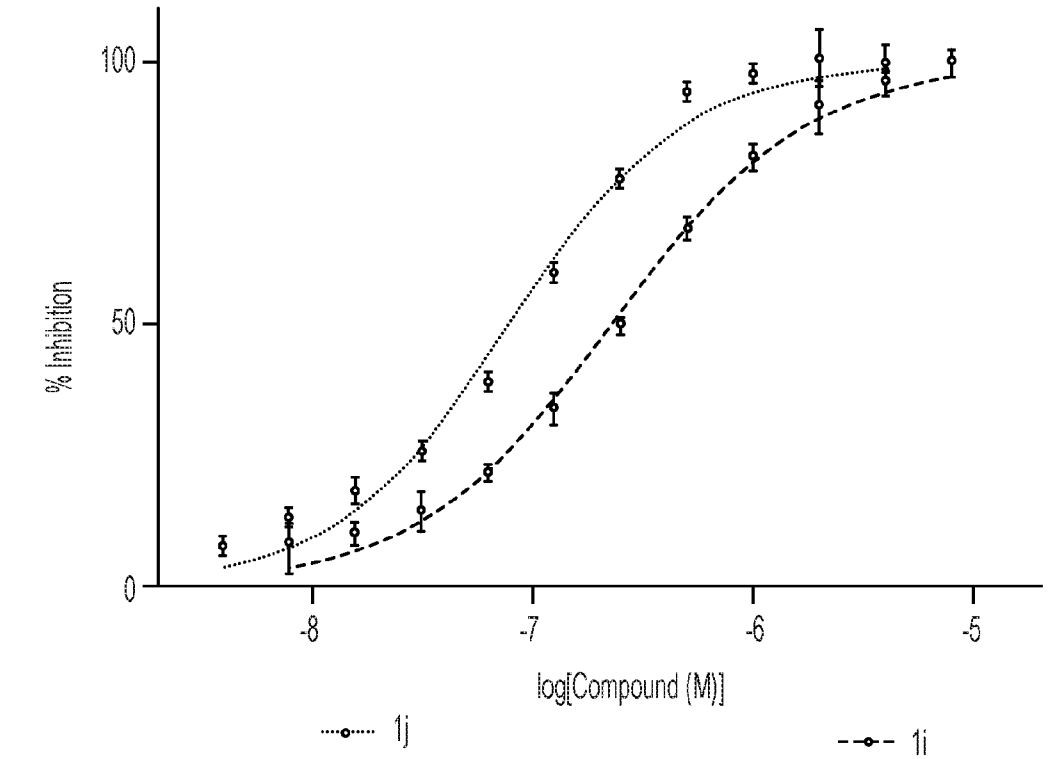

Veolicty (nM/sec)
0
1
2
3
4
10-5.5
10-5
10-4.5
10-4
10-3.5
FRET substrate (M)

Figure 3



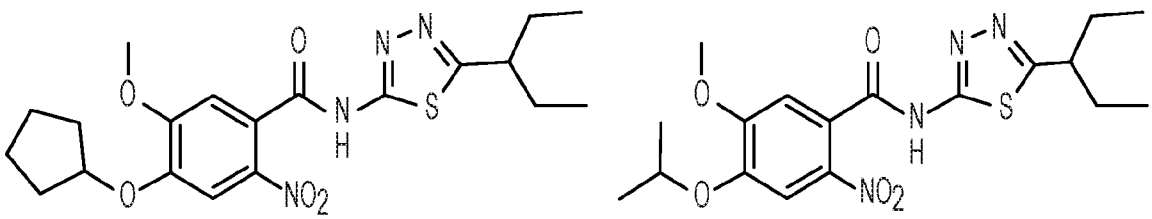


FIG. 3

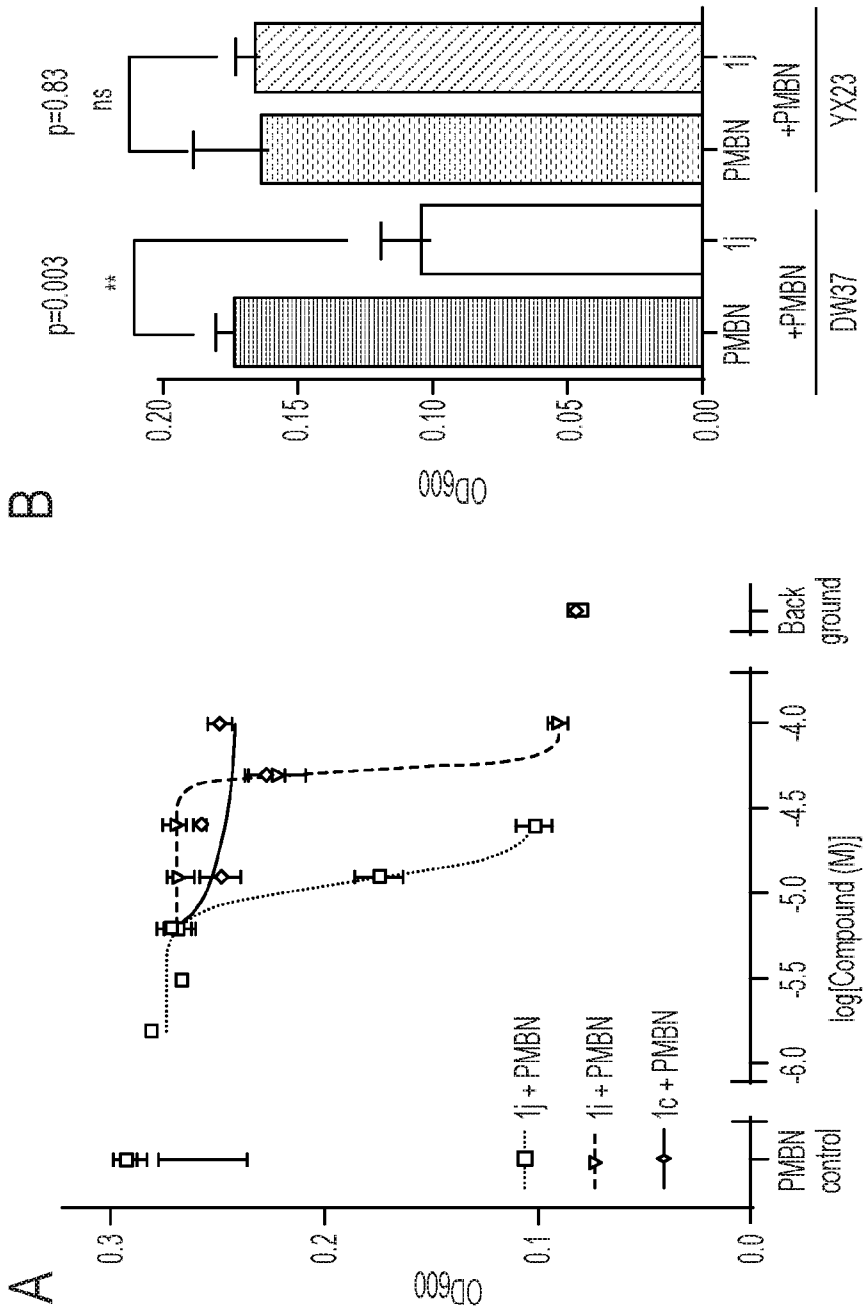

B
p=0.83
ns
p=0.003
**
PMBN
1j
+PMBN
YX23
DW37
0.20
0.15
0.10
0.05
0.00
OD600
A
0.3
0.2
0.1
0.0
1j + PMBN
1i + PMBN
1c + PMBN
PMBN control
-6.0
-5.5
-5.0
-4.5
-4.0
log[Compound (M)]
Back ground

Figure 4

Figure 5

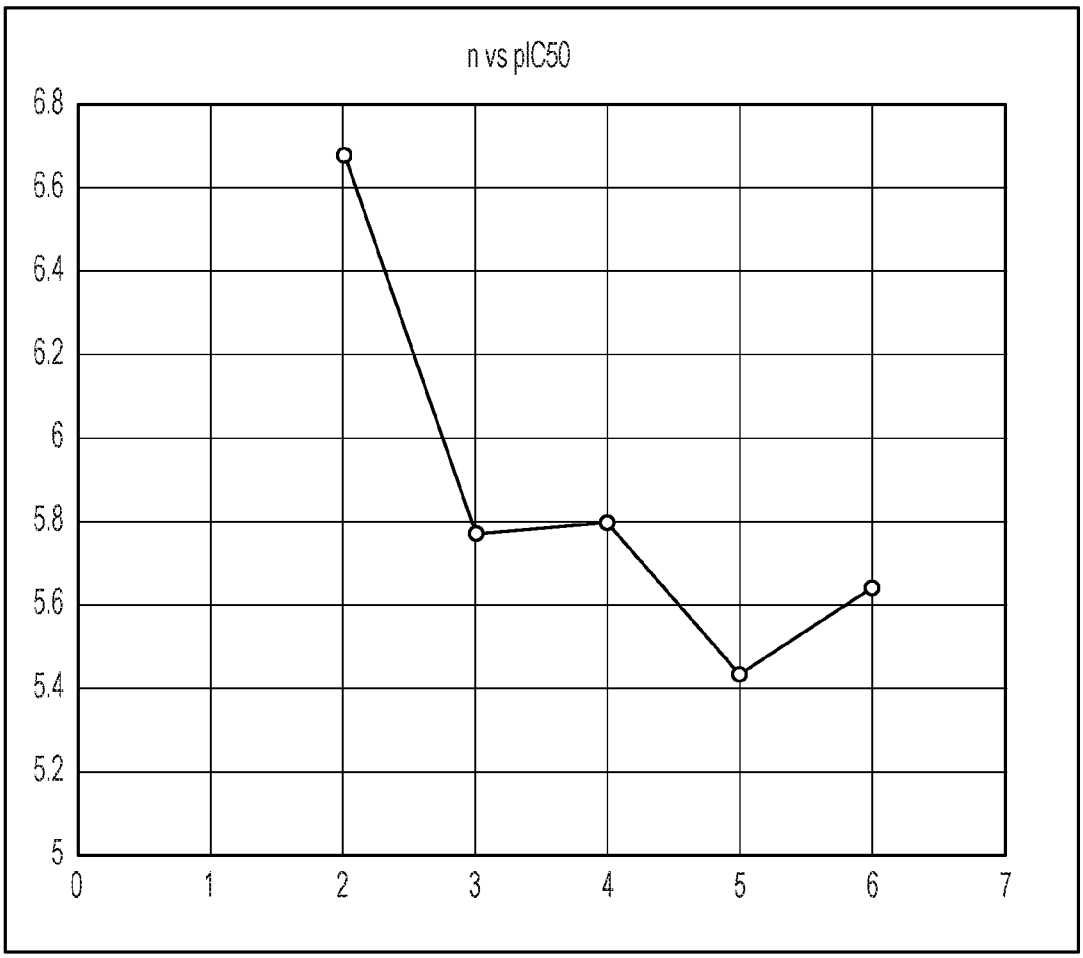
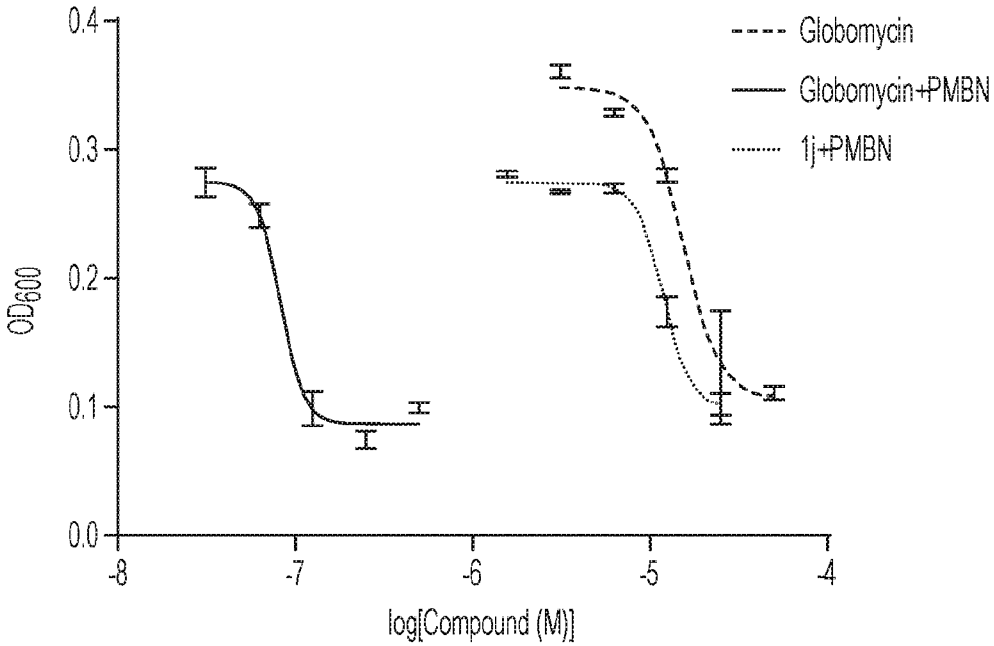


Figure 6


0.4
0.3
0.2
0.1
0.0
OD600
-8
-7
-6
-5
-4
log[Compound (M)]
Globomycin
Globomycin+PMBN
1j+PMBN

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/40693

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17; A61K 39/395; A61P 35/00 (2018.01)
CPC - A61K 2039/507; A61K 2300/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/040762 (The Scripps research institute) 09 March 2017 (09.03.2017) entire document especially abstract; para [0007]; figure 12	1, 3-6
X	Pubchem-CID 27297006, Create Date: 28 May 2009 (28.05.2009), page 3	1-2
X	Pubchem-CID 803244, Create Date: 08 July 2005 (08.07.2005), page 3	1-4
X	Pubchem-CID 1294114, Create Date: 10 July 2005 (10.07.2005), page 3	1-4
X	Pubchem-CID 825343, Create Date: 09 July 2005 (09.07.2005), page 3	1-4
X	Pubchem-CID 28389959, Create Date: 28 May 2009 (28.05.2009), page 3	1-4

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2018	05 NOV 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/40693

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims 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. ☐ Claims 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:
--please see supplemental box --

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 claims Nos.:

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 claims Nos.:
claims 1-6

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/40693

Box III:lack of unity

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: Claims 1-6 drawn towards a compound of formula I

Group II: Claims 7-8 drawn towards a method of screening compounds for inhibitory bioactivity of a bacterial lipoprotein signal peptidase (Lsp), comprising contacting a Lsp peptide FRET substrate, comprising a hexapeptide VTGCAK, with a N-terminal dabsyl quencher and C-terminal EDANS fluorophore, wherein the cysteine residue of the hexapeptide is S-alkylated with a diacylglycerol residue, and a candidate inhibitor compound, then measuring fluorescence from the fluorophore signalling cleavage of the Lsp FRET substrate and its inhibition by the candidate inhibitor.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I requires a compound of formula I, which is not required by Group II.

Group II requires a method of screening compounds for inhibitory bioactivity of a bacterial lipoprotein signal peptidase (Lsp), comprising contacting a Lsp peptide FRET substrate, comprising a hexapeptide VTGCAK, with a N-terminal dabsyl quencher and C-terminal EDANS fluorophore, wherein the cysteine residue of the hexapeptide is S-alkylated with a diacylglycerol residue, and a candidate inhibitor compound, then measuring fluorescence from the fluorophore signalling cleavage of the Lsp FRET substrate and its inhibition by the candidate inhibitor, which is not required by Group I.

Shared Technical Features:

There is no shared technical feature between groups I and II

Therefore, the inventions of Group I-II thus lack unity under PCT Rule 13