Optimization and Mechanistic Studies of Psammaplin A Type Antibacterial Agents Active against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

K. C. Nicolaou,* Robert Hughes, Jeffrey A. Pfefferkorn, and Sofia Barluenga[a]

Abstract: As described in the preceding article, utilizing a novel combinatorial disulfide exchange strategy, a library of psammaplin A (**1**) analogues was constructed and screened for antibacterial activity leading to the identification of a collection of diverse lead compounds. These combinatorial leads were subsequently refined, through parallel synthesis, to afford a series of highly potent antibacterial agents (e.g. **17**, **57**, **58**, **69**, and **70**), some possessing greater than 50-fold higher activities than the natural product. Evaluation of the selectivity and serum binding properties of some of the most promising compounds and preliminary studies directed at deciphering the mechanism of action of this novel class of antibacterial agents are also included.

Keywords: antibiotics · chemical biology · combinatorial synthesis · psammaplin A · total synthesis

Introduction

In the preceding article,[1] we described a novel combinatorial strategy for rapidly constructing a library of heterodimeric disulfides modeled after the marine-derived antibacterial agent psammaplin A (**1**, Figure 1).[2] Direct screening of this 3828-membered library against a panel of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* strains led to the identification of a number of simple psammaplin A analogues exhibiting superior antibacterial properties to those of the natural product (see **2**–**5**, Figure 1). In view of the renewed emphasis on discovering novel antibiotics[3] and because of the uncertainty as to the structural requirements and mechanism of action of this new class of compounds, we undertook the current studies which were directed at: a) optimization of these initial lead compounds; b) development of a systematic structure–activity relationship (SAR) picture of this class of molecules; and c) preliminary assessment of their chemical mechanism of action. In this paper, we describe details of our studies which led to the discovery of a series of potent antibacterial agents and shed light on the

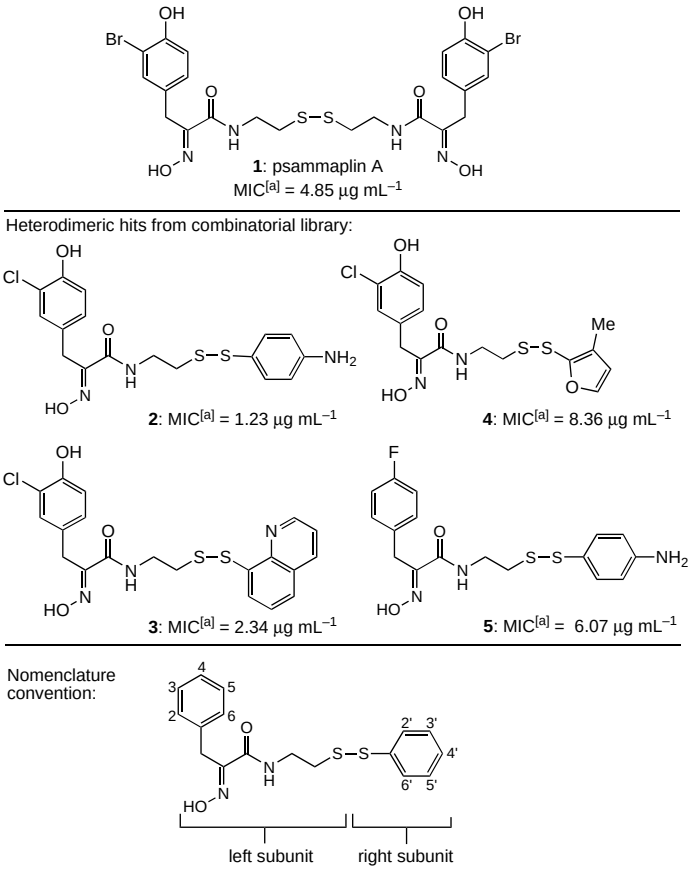

Figure 1. Molecular structure of psammaplin A (**1**), selected lead compounds identified by antibacterial screening of a 3823-membered combinatorial analogue library[1] and nomenclature used throughout the text. [a] MIC is the average for all nine bacterial strains listed in Table 1.

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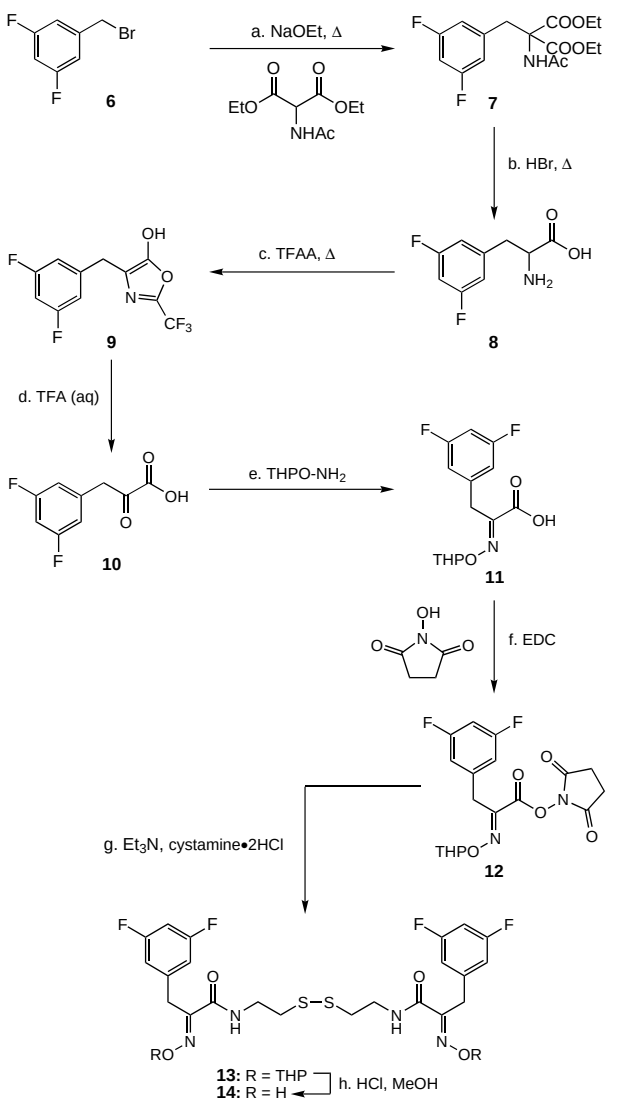
structural requirements for biological activity. Furthermore, and based on preliminary mechanistic studies, we propose a working model for the mechanism of action of this class of antibacterial compounds.

Results and Discussion

Optimization of lead psammaplin A type antibacterial compounds and structure–activity relationships: The identification of several types of structurally simplified, yet highly active lead compounds described in the preceding article[1] left a number of intriguing questions relating to structure–activity relationships pending. In order to gain further insight into these issues, we undertook the molecular design, chemical synthesis, and biological evaluation of a second generation of analogues.

Inspection of the structures of the combinatorial hits **2**–**5** (Figure 1) revealed several trends warranting further investigation. For one, it was apparent that the homodimeric nature of psammaplin A (**1**, Figure 1) was not a prerequisite for biological activity, since half of the molecule could be replaced by either an aromatic or a heteroaromatic subunit (i.e., compounds **2**–**4**, Figure 1). Given the limited data available, however, the structural requirements and optimal substitution of this "right" subunit[4] were not well defined. A second important trend in these structures was suggested by compound **5**, which revealed that the 4-hydroxyl group on the aromatic ring of the "left" subunit of psammaplin A might not be necessary, raising the possibility of simplifying the structure even further while still retaining, or even enhancing activity. Based on these realizations, a two-fold strategy was adopted for optimization of these lead compounds. As shown in Table 1, with the 3-halogen-4-hydroxyl substitution pattern on the "left" subunit held constant, a number of heterodimeric analogues were constructed (employing the chemistry described in ref. [1] and Schemes 1 and 2, see below) to probe the effect of substituents on the other aromatic ring ("right" subunit). Once this region was optimized, it was, in turn, held constant and derivatives with modifications on the "left" subunit were synthesized (see Table 2). Subsequently, several other structural modifications (see Tables 3–5) were also made in an effort to develop a better understanding of the SAR of these compounds. Finally, substitution studies on the disulfide functionality (Tables 6 and 7) were undertaken in an attempt to elucidate this moiety's role in the antibacterial activity of these agents.

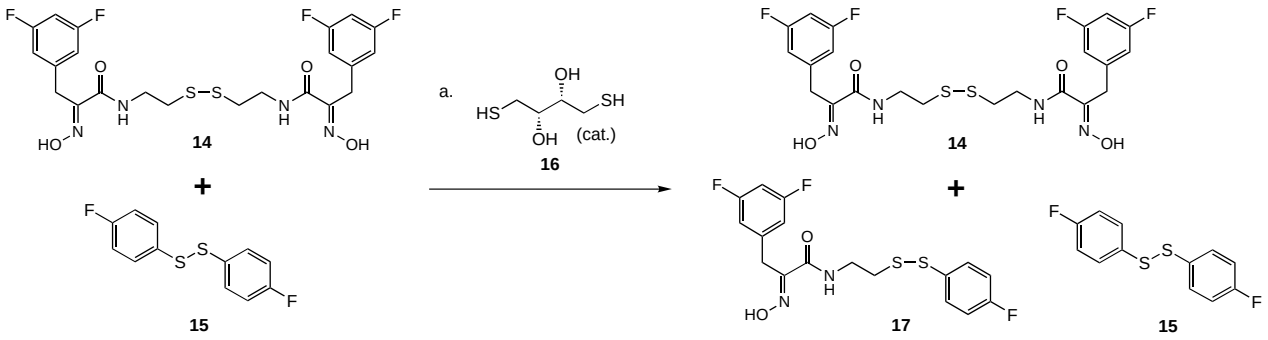


Scheme 1. Synthesis of homodimeric disulfide **14**. a) diethyl acetimidomalonate (1.0 equiv), NaOEt (1.0 equiv), 80 °C, 12 h; b) 48% HBr (excess), 100 °C, 4 h, 70% c) TFAA (excess), 80 °C, 12 h; d) 70% TFA (aq), 23 °C, 24 h; e) THP-ONH_2 (1.5 equiv), EtOH, 23 °C, 12 h; f) NHS (1.9 equiv), EDC (1.7 equiv), 1,4-dioxane, 23 °C, 2 h; g) Et_3N (2.0 equiv), cystamine•2HCl (0.5 equiv), 1,4-dioxane/MeOH 1:1, 23 °C, 12 h; h) HCl (4.0 equiv), CH_2Cl_2/MeOH 20:1, 60 °C, 2 h, 5% over eight steps. EDC = 1-[3-(dimethylamino)propyl)]-3-ethylcarbodiimide; NHS = *N*-hydroxysuccinimide; TFA = trifluoroacetic acid; TFAA = trifluoroacetic anhydride; THP-ONH_2 = *O*-(tetrahydro-2*H*-pyran-2-yl)-hydroxylamine.

All the compounds in Tables 1–5 were synthesized in parallel using the sequence (or part of the sequence) shown in Schemes 1 and 2. Given that each heterodimeric analogue was constructed through the scrambling of the corresponding homodimers (see below), we required access to numerous differentially substituted homodimeric analogues of psammaplin A. These compounds were constructed as illustrated in Scheme 1 for the representative homodimer **14**. Thus, alkylation of diethyl acetimidomalonate with 3,5-difluorobenzyl bromide (**6**) followed by exposure to refluxing HBr afforded racemic 3,5-difluorophenyl alanine (**8**) through a, one-pot, saponification, decarboxylation and deacetylation sequence.[5] The resulting amino acid **8** was refluxed in TFAA (for

Abstract in Greek:

Abstract in Greek: Όπως περιγράφηκε στο προηγούμενο άρθρο, χρησιμοποιώντας μια νέα στρατηγική συνδυαστικής χημείας με εναλλαγή δισουλφιδίων συντέθηκε μια βιβλιοθήκη αναλόγων της ψαμμαπλίνης Α (**1**), και μελετήθηκε βιολογική δραστικότητα τους που οδήγησε στην αναγνώριση μιας σειράς ποικίλων ενώσεων οδηγών. Η σύνθεση των ενώσεων αυτών στη συνέχεια βελτιστοποιήθηκε με παράλληλη σύνθεση για να δώσει μια σειρά από πολύ ισχυρές αντιβιοτικές ενώσεις (π.χ. **17**, **57**, **58**, **69** και **70**), με κάποιες να εμφανίζουν μέχρι και μεγαλύτερη από 50 φορές δραστικότητα σε σχέση με το φυσικό προϊόν. Στο παρόν κείμενο περιγράφονται επίσης η εκτίμηση της εκλεκτικότητας και της ικανότητας δέσμευσης με τον ορό κάποιων από τις πιό πολλά υποσχόμενων ενώσεων και πρωταρχικές μελέτες με στόχο την αποκρυπτογράφηση του μηχανισμού δράσης αυτής της νέας τάξης αντιβακτηριακών παραγόντων.



Scheme 2. Scrambling of homodimeric disulfides to produce heterodimeric analogues. a) Dithiothreitol (0.15 equiv), **16** (1.0 equiv), THF/K_2HPO_4 3:1 (pH 8.3), 23 °C, 12 h, 34 % (of **17**).

abbreviations of reagents and protecting groups, see legends in schemes) to form trifluoromethyl oxazolone **9**, which was distilled from the reaction vessel and hydrolyzed with aqueous TFA to provide α-ketoacid **10**. The latter compound **10** was then condensed with *O*-(tetrahydro-2*H*-pyran-2-yl)-hydroxylamine to afford oxime **11**, which was coupled with cystamine [$(H_2NCH_2CH_2S)_2$] through the intermediacy of the corresponding succinimate ester. Hence, oxime carboxylic acid **11** was treated with *N*-hydroxysuccinimide in the presence of EDC to give succinimate ester **12**, which was immediately treated with cystamine and Et_3N to provide, through a bis-coupling reaction, amide **13**. Finally, the THP protecting groups on the oximes were removed (HCl, MeOH, 60 °C), furnishing homodimer **14** in 5 % overall yield over eight steps. It is noteworthy that over this eight-step sequence, only a single chromatographic purification (that of the final compound) was necessary, thus enabling rapid parallel synthesis of the requisite homodimeric starting materials (see Tables 3 – 5).

With these homodimers available, the desired heterodimeric analogues were synthesized as shown in Scheme 2 for representative compound **17** using the same disulfide exchange chemistry employed for the synthesis of the original combinatorial library.[1] Thus, the two constituent homodimers **14** and **15** were mixed in the presence of catalytic amounts of dithiothreitol (**16**) to afford a 1:2:1 mixture of compounds (**14**:**17**:**15**), which were readily separated by preparative thin-layer chromatography to ultimately afford heterodimer **17** in 34 % yield (50 % theoretical yield). This procedure was repeated in parallel for all heterodimeric analogues shown in Tables 1 – 5.

Analogues with substitutions on the "right" subunit: As shown in Table 1, the first region of the molecule to be examined for its SARs was the aryl or heteroaryl, "right" subunit found in all of the combinatorial hits. Thus, maintaining the 3-chloro-4-hydroxyl substitution pattern constant on the "left" side of the molecule, a series of aryl heterodimers (**2**, **18** – **30**, Table 1) bearing amines, alkyl groups, halogens, hydroxyls, ethers, and nitro groups at various positions on the "right" ring system were constructed. Biological screening of this series of analogues revealed that the most active heterodimers (i.e., MIC $\leq 2\ \mu g\,mL^{-1}$) were those containing the following substituents: 4′-NH_2, 4′-Me, 4′-F, 4′-Cl, or 4′-OH, thereby suggesting the importance of substitution at 4′-position on this ring. This trend was reinforced by examination of compounds **2**, **18**, and **19** containing 4′-NH_2, 3′-NH_2, and 2′-NH_2 substituents, respectively, since the rank order of antibacterial activity of these compounds was **2** > **18** > **19**. While these 4′-position substituents (i.e., 4′-NH_2, 4′-Me, 4′-F, 4′-Cl, or 4′-OH) were electronically diverse, they were isosteric, suggesting that sterics is perhaps the governing factor at this position.

Analogues with substitutions on the "left" subunit: As demonstrated with compounds **31** – **35** (Table 1) several variants of the 3-chloro-4-hydroxyl moiety were next prepared, including 3-bromo-4-hydroxyl (**31**), 3-iodo-4-hydroxyl (**32** and **33**), and 3-hydroxyl (**34** and **35**). While the 3-bromo-4-hydroxyl compound **31** was equipotent with its 3-chloro-4-hydroxyl counterpart (compound **2**), the iodo variants proved to be less active than their chloro counterparts. Finally, compounds **34** and **35** wherein the phenolic hydroxyl was moved from the 4-position to the 3-position on the "left" aromatic unit lost significant potency, indicating that the 3-halogen and/or 4-hydroxyl were essential for the potent antibacterial activity of this class of compounds.

In order to follow up this latter observation, a series of analogues focusing specifically on the "left" subunit were designed and synthesized as shown in Table 2. These designed compounds sought to determine the role of the phenol as well as the optimal nature (i.e., F, Cl or Br) and position (2, 3, or 4) of the halogen substituent. Compounds **42** and **43**, which contain no substituents on the aromatic ring, were four-fold and 10-fold less active, respectively, than their 3-chloro-4-hydroxyl counterparts (compounds **2** and **15**, Table 1) confirming that some degree of aromatic substitution on the "left" side was necessary for optimal activity. Interestingly, however, it was quickly discovered that the phenolic group was not a prerequisite for potent antibacterial activity, as suggested by compounds **46** – **52**, all of which contain only a 3-fluoro substituent on the aryl ring of the "left" subunit. In general, these compounds are of equal or greater potency than the corresponding compounds with a 3-halogen-4-hydroxyl substitution pattern on the "left" aryl ring system. Further studies revealed that halogen substitution at the 3-position was preferred to substitution at the 4-position as demonstrated by the 3-fluoro compound **46** (Table 2) which was more potent than the 4-fluoro compound **5** (Table 2).

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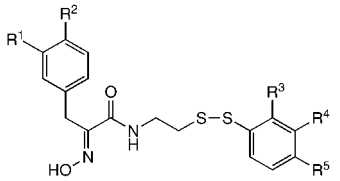
Table 1. Antibacterial activity (MIC/μg mL^{-1}) of heterodimers with varied aromatic substituents.



	R^1	R^2	R^3	R^4	R^5	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
2	Cl	OH	H	H	NH_2	1.56	0.19	1.56	1.56	3.12	0.78	0.78	0.78	0.78	1.22	1.25
18	Cl	OH	H	NH_2	H	6.25	1.56	6.25	3.12	12.5	3.12	6.25	6.25	6.25	4.30	6.87
19	Cl	OH	NH_2	H	H	12.5	0.39	12.5	6.25	25	3.12	6.25	6.25	6.25	7.91	9.37
20	Cl	OH	H	H	NMe_2	3.12	0.78	6.25	6.25	6.25	1.56	3.12	6.25	3.12	4.10	4.06
21	Cl	OH	H	H	H	6.25	0.39	3.12	3.12	12.5	1.56	3.12	1.56	3.12	3.22	4.37
22	Cl	OH	H	H	Me	3.12	0.39	1.56	1.56	12.5	1.56	1.56	1.56	3.12	1.66	4.06
23	Cl	OH	H	Cl	H	6.25	0.39	6.25	3.12	12.5	0.78	1.56	1.56	1.56	4.00	3.59
24	Cl	OH	H	H	Cl	3.12	0.19	1.56	3.12	6.25	1.56	6.25	0.78	1.56	2.00	3.28
25	Cl	OH	H	H	F	3.12	0.39	1.56	3.12	12.5	1.56	0.78	1.56	3.12	2.05	3.91
26	Cl	OH	H	H	OMe	6.25	0.78	3.12	6.25	12.5	1.56	0.78	3.12	6.25	4.16	4.84
27	Cl	OH	H	H	OH	3.12	0.78	1.56	0.78	6.25	1.56	0.78	1.56	1.56	1.56	2.34
28	Cl	OH	H	Cl	Cl	12.5	0.19	6.25	12.5	25	3.12	6.25	0.78	6.25	7.86	8.28
29	Cl	OH	NH_2	H	Cl	12.5	0.19	6.25	12.5	12.5	1.56	6.25	1.56	3.12	7.86	5.00
30	Cl	OH	H	H	NO_2	6.25	0.09	6.25	6.25	12.5	3.12	3.12	3.12	3.12	4.71	5.00
31	Br	OH	H	H	NH_2	3.12	0.39	0.78	1.56	1.56	0.78	0.78	0.78	0.39	1.46	0.86
32	I	OH	H	H	F	6.25	0.78	6.25	3.12	12.5	1.56	3.12	3.12	6.25	4.10	5.31
33	I	OH	H	H	Cl	6.25	0.39	6.25	3.12	12.5	0.78	1.56	1.56	3.12	4.10	3.91
34	OH	H	H	H	F	50	3.12	25	25	50	12.5	50	25	50	25.8	37.8
35	OH	H	H	H	Cl	12.5	1.56	12.5	6.25	25	3.12	12.5	12.5	25	8.20	15.6
1	psammaplin A					6.25	3.12	6.25	6.25	6.25	3.12	3.12	6.25	3.12	5.47	4.37
36	dichloropsammaplin A					6.25	3.12	6.25	6.25	6.25	3.12	3.12	3.12	6.25	5.47	4.37
37	ciprofloxacin					3.12	0.05	0.19	0.19	25	0.19	25	25	25	0.89	20.0
38	vancomycin					0.78	0.19	0.78	1.56	1.56	0.78	12.5	1.56	3.12	0.83	3.90
39	penicillin G					<0.05	<0.05	0.19	<0.05	25	0.78	3.12	6.25	12.5	<0.05	9.53
40	tetracycline					0.09	0.09	0.78	0.09	50	0.78	1.56	1.56	1.56	0.26	11.0
41	erthyromycin					0.09	0.39	0.09	0.19	>50	>50	0.78	25	25	0.19	>50

[a] *Staphylococcus aureus* ATCC 6538. [b] *Staphylococcus aureus* ATCC 13709. [c] *Staphylococcus aureus* ATCC 29213. [d] *Staphylococcus aureus* ATCC 25923. [e] *Staphylococcus aureus* ATCC 700698, resistant to methicillin and heterogeneous susceptibility to vancomycin. [f] *Staphylococcus aureus* ATCC 43300, resistant to methicillin. [g] *Staphylococcus aureus* ATCC 700787, resistant to methicillin and intermediate susceptibility to vancomycin. [h] *Staphylococcus aureus* ATCC 700788, resistant to methicillin and intermediate susceptibility to vancomycin. [i] *Staphylococcus aureus* ATCC 700789, resistant to methicillin and intermediate susceptibility to vancomycin. [j] Average MIC calculated from four strains of *Staphylococcus aureus* (6538, 13709, 29213 and SA 25923). [k] Average MIC calculated from five strains of methicillin-resistant *Staphylococcus aureus* (700698, 43300, 700787, 700788, and 700789).

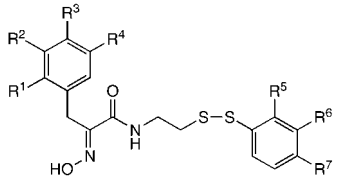
Examination of all these halogenated compounds revealed that the two most fruitful substitution patterns for antibacterial activity were the 3,5-difluoro (**17** and **55**) and the 3-bromo (**57** and **58**). The most potent compound identified through these studies (**57**) was composed of a 3-bromophenyl alanine "left" subunit linked to a 4′-aminophenyl "right" subunit, providing a heterodimer with an average MIC value of 0.09 μg mL^{-1} against both SA and MRSA strains.

Analogues with heteroaromatic "right" subunits: So as not to overlook the potential benefit of using a heteroaromatic aryl ring as the "right" subunit, a limited number of heteroaryl derivatives were synthesized as shown in Table 3. Of these compounds, furan **63** and quinyl **64** exhibited antibacterial activities comparable to the several of the more potent compounds found previously (see Table 1 and Table 2). Unfortunately, both compounds **63** and **64** exhibited substantial cytotoxicity against non-bacterial cell lines (see below) thereby discouraging further pursuit of this series.

Analogues with sterically hindered "right" subunits: Before leaving the discussion on the nature and optimal substitution pattern for the two aryl rings found in all active analogues, we should mention another notable series of rather unusual, yet quite active heterodimers, those containing a bulky substituent as their "right" subunit (see Table 4). Compounds containing a 2′,6′-dimethylphenyl subunit (i.e., **68**–**70**, see Table 4) exhibited potent antibacterial activities, with the 3,5-difluorophenyl alanine (**69**) and 3-bromophenyl alanine (**70**) species being the most active with average MIC values of 0.27 and 0.11 μg mL^{-1}, respectively.

Probing the functional importance of the oxime moiety: An additional region of SAR interest was the α-oxime motif

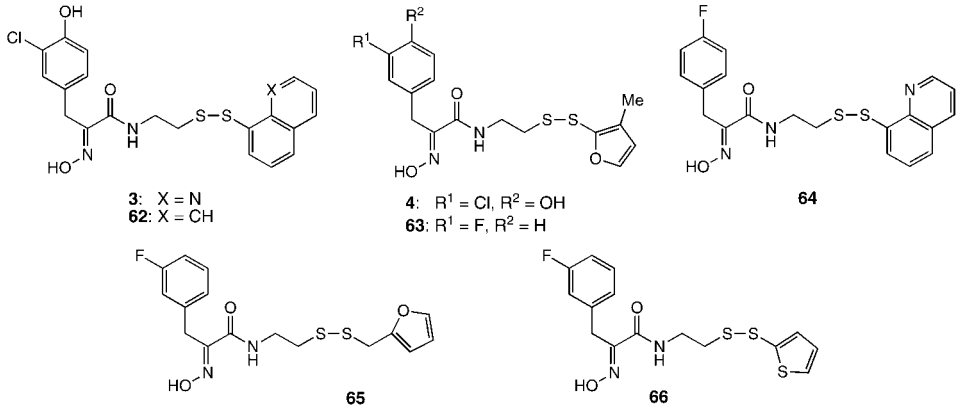
Table 2. Antibacterial activity (MIC/μg mL^{-1}) of heterodimers with varied aromatic substituents.



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
42	H	H	H	H	H	H	F	6.25	0.39	6.25	6.25	12.5	1.56	1.56	3.12	3.12	4.79	4.37
43	H	H	H	H	H	H	NH_2	12.5	3.12	12.5	12.5	25	6.25	6.25	12.5	12.5	10.2	12.5
44	F	H	H	H	H	H	NH_2	6.25	1.56	12.5	6.25	12.5	6.25	3.12	3.12	3.12	6.64	5.62
45	F	H	H	H	H	H	F	12.5	0.09	12.5	6.25	12.5	3.12	3.12	12.5	6.25	7.83	7.50
46	H	F	H	H	H	H	NH_2	1.56	0.78	1.56	1.56	1.56	0.78	0.78	0.78	0.78	1.36	0.94
47	H	F	H	H	H	H	NH_2	1.56	0.78	0.78	1.56	1.56	0.78	0.78	0.78	0.78	1.17	0.94
48	H	F	H	H	F	H	H	0.78	0.19	0.39	0.78	1.56	0.39	0.19	0.39	0.39	0.54	0.58
49	H	F	H	H	H	H	F	0.78	0.19	0.39	0.78	1.56	0.39	0.19	0.39	0.39	0.54	0.58
50	H	F	H	H	Cl	H	H	3.12	0.09	3.12	3.12	3.12	0.39	0.78	0.78	0.78	2.39	1.17
51	H	F	H	H	H	Cl	H	1.56	0.09	1.56	3.12	3.12	0.39	0.39	0.39	0.78	1.58	1.01
52	H	F	H	H	H	H	Cl	1.56	0.39	1.56	1.56	6.25	0.78	0.78	0.78	1.56	1.27	2.03
53	H	H	F	H	H	H	F	1.56	0.39	1.56	1.56	6.25	0.39	0.78	0.78	0.78	1.27	1.80
54	H	H	F	H	H	H	Cl	3.12	3.12	3.12	6.25	6.25	3.12	3.12	3.12	3.12	3.90	3.75
5	H	H	F	H	H	H	NH_2	6.25	1.56	12.5	6.25	12.5	6.25	3.12	3.12	3.12	6.64	5.62
17	H	F	H	F	H	H	F	0.78	0.09	0.78	0.78	0.78	0.19	0.19	0.39	0.19	0.61	0.35
55	H	F	H	F	H	H	Cl	1.56	0.78	0.78	1.56	1.56	0.19	0.39	0.39	0.39	1.17	0.58
56	H	H	Cl	H	H	H	Cl	1.56	0.78	1.56	1.56	3.12	0.78	0.78	0.78	0.78	1.37	1.25
57	H	Br	H	H	H	H	NH_2	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
58	H	Br	H	H	H	H	F	0.09	0.09	0.09	0.19	0.19	0.09	0.09	0.09	0.09	0.12	0.11
59	H	H	Br	H	H	H	NH_2	6.25	3.12	6.25	6.25	12.5	6.25	3.12	3.12	3.12	5.46	5.62
60	H	H	Br	H	H	H	F	3.12	0.78	3.12	1.56	6.25	0.78	0.78	0.78	0.78	2.14	1.87
61	H	H	Br	H	H	H	Cl	3.12	1.56	3.12	6.25	6.25	1.56	6.25	3.12	1.56	3.51	3.75

See Table 1, footnotes ([a]–[k]) for bacterial strain information.

Table 3. Antibacterial activity (MIC/μg mL^{-1}) of heterodimers containing heteroaromatic units.



3: X = N
62: X = CH

4: R^1 = Cl, R^2 = OH
63: R^1 = F, R^2 = H

64

65

66

	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
62	12.5	0.78	6.25	12.5	25	6.25	3.12	3.12	6.25	8.00	8.75
3	3.12	0.39	3.12	3.12	3.12	0.39	1.56	3.12	3.12	2.44	2.26
4	12.5	0.39	6.25	3.12	25	6.25	6.25	3.12	12.5	5.56	10.6
63	0.78	0.19	0.39	0.78	0.78	0.39	0.19	0.39	0.39	0.54	0.43
64	1.56	0.78	0.78	1.56	3.12	1.56	0.78	1.56	1.56	1.17	1.72
65	12.5	3.12	12.5	12.5	6.25	6.25	6.25	6.25	6.25	10.2	6.25
66	6.25	0.19	6.25	6.25	6.25	1.56	3.12	3.12	3.12	4.73	3.43

See Table 1, footnotes ([a]–[k]) for bacterial strain information.

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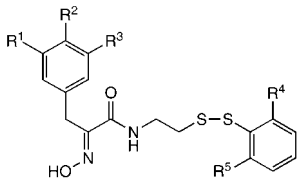
Table 4. Antibacterial activity (MIC/μg mL^{-1}) of 2,6-disubstituted phenyl heterodimers.



	R^1	R^2	R^3	R^4	R^5	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
67	Cl	OH	H	Cl	Cl	6.25	0.78	6.25	3.12	12.5	1.56	3.12	3.12	6.25	4.10	5.31
68	Cl	OH	H	Me	Me	1.56	0.39	1.56	0.78	3.12	0.39	0.39	0.78	0.78	1.11	1.09
69	F	H	F	Me	Me	0.39	0.19	0.19	0.39	0.39	0.19	0.19	0.19	0.39	0.29	0.27
70	Br	H	H	Me	Me	0.09	0.09	0.09	0.19	0.19	0.09	0.09	0.09	0.09	0.12	0.11

See Table 1, footnotes ([a]–[k]) for bacterial strain information.

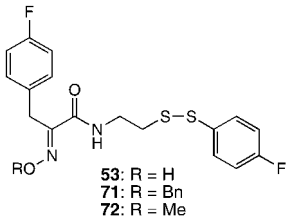
found in psammaplin A and all active heterodimers considered in these studies. As shown in Table 5, alkylation of the hydroxyl functionality of this oxime to give either a methyl oxime (e.g. **71**) or a benzyl oxime (e.g. **72**) resulted in loss of activity, suggesting the importance of the free hydroxyl group at this position.

Probing the functional importance of the disulfide moiety: A final set of SAR studies focused on the intriguing question of the importance of the central disulfide moiety. While this unique structural feature of psammaplin A (**1**, Figure 1) allowed implementation of our original combinatorial scrambling strategy, it remained to be determined as to whether or not its presence was required for biological activity. This question was addressed through the synthesis of several homo- and heterodimeric molecules which lacked the disulfide functionality. Thus, the carbon analogue (**73**, Table 6) of dichloro-psammaplin A (**36**, Table 6) was synthesized using a procedure identical to that described previously in Scheme 1, with the exception that 1,6-diaminohexane was substituted for cystamine. Interestingly, when assayed, this carbon analogue exhibited only a low level of activity against a few strains and was completely inactive against others (see Table 6). On average, **73** was found to be more than 10-fold less active than its disulfide counterpart, compound **36**. Additional evidence for the importance of the intact disulfide unit was found when the reduced form of dichloro-psammaplin A (i.e., thiol **74**, Table 6) was synthesized and assayed revealing its insignificant antibacterial profile.

We next undertook a similar study for one of the heterodimeric analogues. Disulfide **21** (see Table 7), consisting of phenyl sulfide linked to the 3-chloro-4-hydroxyl subunit, was selected for these studies based on synthetic accessibility considerations. Two isomeric thioether analogues (compounds **82** and **84**) as well as the all-carbon analogue (**87**) of disulfide **21** (Table 1) were synthesized as outlined in Scheme 3.

The two thioether-containing primary amine coupling partners **76** and **78** were prepared by S-alkylation of thiophenol with 2-bromoethylamine hydrochloride and S-alkylation of 2-aminoethanethiol hydrochloride with benzyl bromide, respectively. Coupling of amines **76**, **78**, and **85** with carboxylic acid **79** was accomplished through the intermediacy of the succinimate ester **80** to afford amides **81**, **83**, and **86**, respectively. Finally, the THP protecting groups of the oximes were removed under acidic conditions (HCl, MeOH, 60 °C) to afford analogues **82**, **84**, and **87**. These compounds were then assayed for antibacterial activity and the results are shown in Table 7. As was the case with homodimer **73** (Table 6), all compounds lacking the intact disulfide moiety were at least 10-fold less active than the parent disulfide-containing compound (**21**, Table 1). Together, these non-disulfide containing homo- and heterodimers provided convincing evidence that the disulfide motif plays a critical role in the biological activity of these compounds. Interestingly, however, while

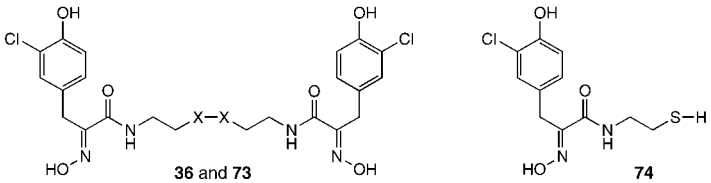
Table 5. Antibacterial activity (MIC/μg mL^{-1}) of heterodimers with derivatized oximes.



	R	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
53	H	1.56	0.39	1.56	1.56	6.25	0.39	0.78	0.78	0.78	1.27	1.80
71	Bn	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
72	Me	>50	6.25	>50	50	>50	12.5	12.5	>50	>50	>50	>50

See Table 1, footnotes ([a]–[k]) for bacterial strain information.

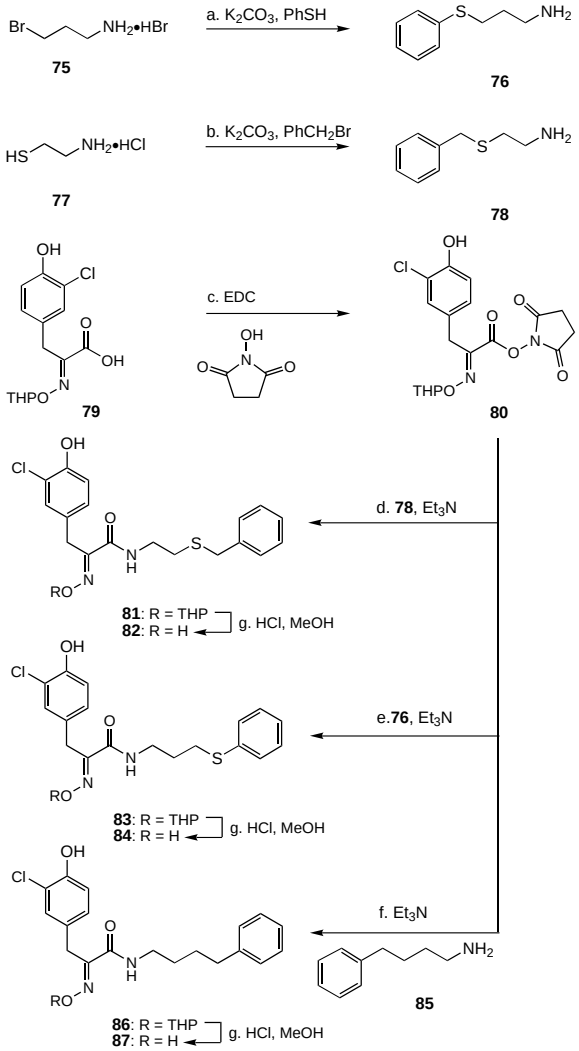
Table 6. Antibacterial activity (MIC/μg mL^{-1}) of dichloro-psammaplin A (**36**) and non-disulfide analogues.



36 and **73** **74**

	X	Y	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
36	S	S	6.25	3.12	6.25	6.25	6.25	3.12	3.12	6.25	3.12	4.68	4.37
73	CH_2	CH_2	25	>50	50	>50	50	>50	>50	>50	>50	>50	>50
74	-	-	>50	25	>50	>50	>50	>50	>50	>50	>50	>50	>50

See Table 1, footnotes ([a]–[k]) for bacterial strain information.



Scheme 3. Synthesis of non-disulfide analogues **82**, **84**, and **87**. a) 3-bromopropylamine hydrobromide (1.0 equiv), PhSH (1.0 equiv), K_2CO_3 (2.0 equiv), EtOH, 80 °C, 12 h, 35%; b) 2-aminoethanethiol hydrochloride (1.0 equiv), $PhCH_2SH$ (1.0 equiv), K_2CO_3 (2.0 equiv), EtOH, 80 °C, 12 h, 90%; c) NHS (1.9 equiv), EDC (1.7 equiv), 1,4-dioxane, 23 °C, 2 h; d) Et_3N (2.0 equiv), **78** (1.0 equiv), 1,4-dioxane/MeOH 1:1, 23 °C, 12 h; e) Et_3N (2.0 equiv), **76** (1.0 equiv), 1,4-dioxane/MeOH 1:1, 23 °C, 12 h; f) Et_3N (2.0 equiv), **85** (1.0 equiv), 1,4-dioxane/MeOH 1:1, 23 °C, 12 h; g) HCl (4.0 equiv), CH_2Cl_2/MeOH 20:1, 60 °C, 2 h, 36% over five steps.

the potency of a given compound is significantly (> 10-fold) diminished when its disulfide is replaced, in most cases such non-disulfide analogues do retain some antibacterial activities (with MIC values in the range of 25–50 μg mL^{-1}). A discussion of the potential mechanistic implications of these findings will be presented in a separate section below.

As a whole, these SAR studies served to identify a collection of novel heterodimeric disulfides (see representative compounds **17**, **57**, **58**, **69**, and **70**, Figure 2) with more potent antibacterial activities than psammaplin A (**1**, Figures 1 and 2) and the original combinatorial lead compounds (**2**–**5**, Figure 1). These SAR studies also served to effectively elucidate which structural features of this new class of antibacterial agents are required for biological activity as summarized in Figure 2. These findings led to the following conclusions: a) the symmetrical nature of psammaplin A is not required for activity and, in fact, the second subunit can be replaced with a single aromatic or heteroaromatic unit; and b) the potencies of these latter heterodimers are significantly dependent upon the substitution patterns on this single aromatic system, with optimal substituents including 4′-NH_2, 4′-F, and 2′,6′-dimethyl; c) the original 4-hydroxyl functionality of the "right" subunit is not needed, but halogenation of this subunit in either the 3- or 3,5-position(s) is required; d) the free oxime is essential for biological activity; e) substitution of the disulfide moiety (S-S) with structurally equivalent replacements (e.g. CH_2-CH_2, CH_2-S, or S-CH_2) resulted in a >10-fold loss in activity. In order to further evaluate these compounds with regards to their potential as therapeutic candidates and gain insights into their mechanism of action, we next undertook a series of more detailed biological studies.

In vitro toxicity and serum binding properties of selected compounds: Studies aimed at determining the degree of cytotoxicity and selectivity of the newly discovered antibacterial agents, employing a toxicity assay and a serum binding assay, were conducted. Preliminary in vitro toxicities of several of these compounds were estimated by measuring their IC_{50} values against fibroblast and lymphocyte cell lines.[6] The average IC_{50} [μg mL^{-1}] value of a given compound in these two cell lines was then divided by its average MIC value [μg mL^{-1}] against nine SA and MRSA strains (see Tables 1–7) to afford a therapeutic index (TI) ratio, which served as a

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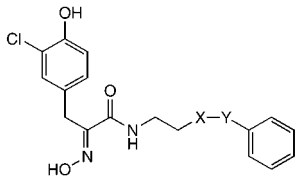
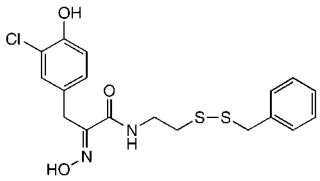
Table 7. Antibacterial activity (MIC/μg mL^{-1}) of heterodimer (**21**) and selected non-disulfide analogues.



21, **82**, **84**, and **87**



88

	X	Y	SA[a] 6538	SA[b] 13709	SA[c] 29213	SA[d] 25923	MRSA[e] 700698	MRSA[f] 43300	MRSA[g] 700787	MRSA[h] 700788	MRSA[i] 700789	AVG[j] SA	AVG[k] MRSA
21	S	S	3.12	0.39	1.56	1.56	12.5	1.56	1.56	1.56	1.56	1.65	3.75
82	S	CH_2	50	50	25	50	50	50	50	50	50	44	50
84	CH_2	S	50	25	25	50	25	25	25	25	50	38	30
87	CH_2	CH_2	25	25	25	25	25	25	25	50	25	25	30
88	-	-	3.12	1.56	1.56	6.25	6.25	3.12	1.56	3.12	3.12	4.69	3.43

See Table 1, footnotes ([a]–[k]) for bacterial strain information.

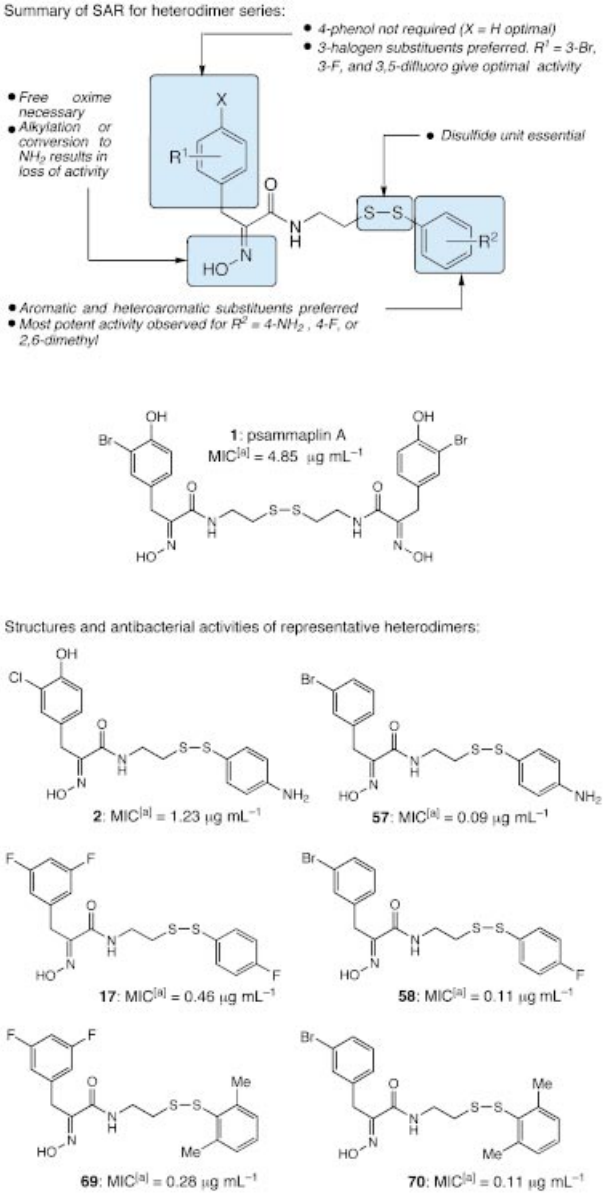


Figure 2. Summary of most active heterodimeric compounds. Minimum structural requirements and SAR for antibacterial activity of heterodimeric psammaplin A analogues. [a] MIC is the average of all nine bacterial strains listed in Table 1.

rough estimate of the selectivity of these agents. As shown in Table 8, the natural product, psammaplin A (**1**), proved to be rather nonselective in vitro, and, in fact, was more potent against the fibroblasts and lymphocytes than against the bacterial strains. In addition, the early combinatorial leads (compounds **2**–**5**, Figure 1) were found, like psammaplin A (**1**), to be rather nonselective (TI = 2.52, < 0.25, 0.32, and 2.04, respectively) in their actions against bacteria and the selected fibroblast and lymphocyte cell lines. In particular, the quinyl and furyl derivatives (**3** and **4**, Figure 1) were found to be quite toxic, thereby, bringing this vain of investigation to a close. Examination of several later generation compounds, however,

Table 8. Cytotoxicity and selectivity of representative compounds.[a]

	IC_{50} [μg mL^{-1}] fibroblasts[b]	lymphocytes[c]	MIC [μg mL^{-1}] SA[d]	MRSA[e]	TI[f]
1: psammaplin A	2.34	3.12	5.47	4.37	0.56
2	3.12	3.12	1.22	1.25	2.52
3	0.78	<0.39	2.44	2.26	<0.25
4	2.34	3.12	5.56	10.6	0.32
5	18.7	6.25	6.64	5.62	2.04
17	4.69	1.95	0.61	0.35	7.13
24	3.12	1.56	2.00	3.28	0.86
25	2.34	1.56	2.05	3.91	0.63
46	12.5	6.25	1.36	0.94	8.32
47	6.25	3.12	1.17	0.94	4.50
49	4.68	4.68	0.54	0.58	8.32
52	1.95	1.95	1.27	2.03	1.15
53	3.12	4.68	1.27	1.80	2.49
55	3.52	3.12	1.17	0.58	3.94
58	6.25	2.34	0.12	0.11	37.5
63	4.68	6.25	0.54	0.43	11.4
68	1.56	2.34	1.11	1.09	1.77
37: ciprofloxacin	50	15.6	0.89	20.0	2.85

[a] See Table 1–5 for structures of the compounds. [b] Baby hamster kidney, BHK-21 (ATCC CCL10). [c] Jurkat cells. [d] Average MIC [μg mL^{-1}] against the four *Staphylococcus aureus* strains in Table 1. [e] Average MIC [μg mL^{-1}] against the six methicillin-resistant *Staphylococcus aureus* strains in Table 1. [f] Therapeutic Index calculated (average toxicity against lymphocytes and fibroblast)/(average MIC for SA and MRSA).

led to more promising results. Interestingly, it was noted that the 3-halogen-4-hydroxyl compounds (**1**, **2** – **4**, **24**, **25**, and **68**, see Table 8) were generally more toxic than those bearing only halogens (**5**, **17**, **46**, **47**, **49**, **52**, **53**, **58**, and **63**, Table 8). This realization, coupled with the fact that our previous SAR work indicated that the 4-hydroxyl group on the "left" subunit was not necessary for antibacterial activity, suggested the non-phenolic heterodimers as the most promising candidates for further optimization. In fact, the most interesting compound in this study proved to be the heterodimer **58** consisting of a 3-bromo-phenyl alanine subunit and a 4′-fluorophenyl moiety, exhibiting a therapeutic index (TI) of 37.5. While preliminary, these results suggested that it is possible, through structural modification, to separate the general cytotoxic properties of these molecules from their antibacterial properties.

Table 9. Effect of horse serum on antibacterial activity (MIC/μg mL^{-1}) of selected heterodimeric disulfide analogues.[a]

	SA 6538[b]					MRSA 700698[c]				
	none[d]	1 %[e]	3 %[f]	5 %[g]	10 %[h]	none[d]	1 %[e]	3 %[f]	5 %[g]	10 %[h]
1: psammaplin A	6.25	6.25	12.5	25	50	6.25	50	25	50	>50
2	1.56	3.12	6.25	6.25	25	3.12	12.5	12.5	50	50
3	3.12	3.12	6.25	12.5	25	3.12	12.5	12.5	25	25
4	3.12	3.12	3.12	3.12	12.5	6.25	6.25	12.5	12.5	50
5	6.25	6.25	3.12	12.5	50	6.25	6.25	12.5	12.5	50
17	0.19	1.56	3.12	12.5	25	0.78	1.56	12.5	12.5	25
20	3.12	3.12	3.12	3.12	12.5	6.25	6.25	6.25	12.5	12.5
21	6.25	3.12	6.25	25	50	12.5	12.5	12.5	25	25
22	3.12	3.12	6.25	25	25	12.5	12.5	12.5	50	50
25	3.12	6.25	6.25	12.5	50	12.5	12.5	25	25	50
46	0.78	3.12	3.12	6.25	12.5	0.78	1.56	6.25	6.25	25
47	0.78	3.12	12.5	12.5	50	1.56	1.56	25	12.5	25
49	0.39	1.56	6.25	6.25	12.5	1.56	1.56	12.5	12.5	25
52	0.39	3.12	3.12	6.25	12.5	1.56	1.56	6.25	6.25	25
53	3.12	12.5	6.25	25	50	3.12	25	50	25	>50
55	0.19	1.56	3.12	12.5	25	0.39	3.12	25	12.5	25
57	0.09	3.12	6.25	12.5	25	0.09	1.56	6.25	6.25	25
59	0.09	1.56	6.25	6.25	12.5	0.09	0.78	6.25	6.25	12.5
68	0.78	1.56	6.25	12.5	50	0.78	3.12	25	12.5	25
69	0.39	3.12	12.5	12.5	25	0.39	1.56	12.5	6.25	50
70	0.19	3.12	6.25	6.25	12.5	0.19	3.12	6.25	6.25	25
40: tetracycline	0.09	0.09	0.09	0.09	0.09	50	50	50	50	>50

[a] See Table 1 – 5 for structures of the compounds. [b] *Staphylococcus aureus* ATCC 6538. [c] *Staphylococcus aureus* ATCC 700 698, resistant to methicillin and heterogeneous susceptibility to vancomycin. [d] SA 6538 or MRSA 700 698 with no added horse serum. [e] SA 6538 or MRSA 700 698 with 1 % added horse serum. [f] SA 6538 or MRSA 700 698 with 3 % added horse serum. [g] SA 6538 or MRSA 700 698 with 5 % added horse serum. [h] SA 6538 or MRSA 700 698 with 10 % added horse serum.

A second area of interest surrounding this class of compounds was their serum binding properties. It is precedented and accepted that a moderate level of serum binding is important for in vivo efficacy of many therapeutic agents, since this can increase their half-lives by protecting them from metabolism and clearance.[7] At the same time, however, extremely high levels of serum binding can impede a compound's ability to reach its molecular target. With this in mind, we sought to estimate the serum binding properties of selected compounds by re-assaying them for antibacterial activity (against SA 6538 and MRSA 700 698) in the presence of increasing concentrations of horse serum (1, 3, 5, and 10 % by volume). The results as shown in Table 9. In general, all heterodimeric analogues exhibited decreased antibacterial activity in the presence of 10 % serum, indicating significant serum binding at these levels, which was not unexpected given the lipophilic nature of the compounds. The latter property was reflected in the calculated log P values for representative compounds **2**, **20** and **58** which were estimated to be 1.94, 2.81, and 2.89, respectively.[8]

Studies on the mechanism of action of psammaplin A and its analogues: In view of the report[9] claiming inhibition of bacterial DNA gyrase by psammaplin A, we decided to study the action of our compounds on this enzyme. Bacterial DNA gyrase is a member of the topoisomerase family of enzymes which are responsible for the remodeling of DNA topology. Specifically, this enzyme introduces negative supercoils into double stranded DNA, facilitating replication, transcription, and translation.[10] This crucial role of DNA gyrase in bacterial cell function and the lack of a strictly homologous human counterpart, make it an attractive target for the development of antibiotics.[11] In fact, the well-known quinoline and fluoroquinoline antibiotics, including ciprofloxacin (**37**, Figure 3), nalidixic acid, and ofloxacin exert their antibacterial activity by inhibiting this enzyme. Additionally, the coumarin-based antibacterials such as novobiocin (**90**, Figure 3) and chlorobiocin, as well as the natural product cyclothialidine (**89**, Figure 3) also function by blocking DNA gyrase activity.[12] The quinolines, in particular, have found remarkable clinical success, but as with most classes of antibiotics, their widespread use has resulted in the emergence of drug-resistant strains, prompting interest in identifying new classes of gyrase inhibitors. Despite large scale screening efforts, however, relatively few novel small molecule inhibitors have been identified outside of the quinoline and coumarin families.[13]

We first sought to confirm psammaplin A's inhibitory activity against DNA gyrase prior to screening our heterodimeric analogues. A review of the report by Kim et al.[9] revealed that the prior assays of psammaplin A were conducted in the presence of dithiothreitol (DTT). While the reducing agent DTT, utilized to reduce enzyme cross linking, is a standard component of many enzyme assays, its use in the screening of psammaplin A cast a degree of skepticism on these early results, particularly, in light the demonstrated

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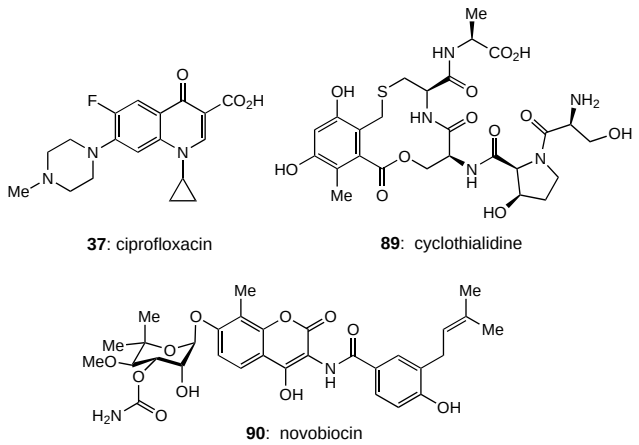

Figure 3. Representative examples of bacterial DNA gyrase inhibitors: ciprofloxacin (**37**), cyclothialidine (**89**) and novobiocin (**90**).

ability of DTT to reduce this disulfide natural product to the corresponding free thiol. It is estimated that under the assay conditions reported by Kim et al. there were more than 10 equivalents of DTT present relative to psammaplin A.[9] HPLC analysis (data not shown) indicated that under this concentration of DTT and at the pH of the assay and over the time-scale of the experiment, the psammaplin A would have been substantially reduced to the corresponding free thiol. It is, therefore, questionable whether the IC_{50} values previously reported accurately reflect the activity of psammaplin A. In fact, these experimental conditions may explain why these authors observed a relatively low IC_{50} value (approximately 50 $\mu g\,mL^{-1}$) for psammaplin A as an inhibition of this enzyme given that the compound has an average MIC of 4.85 $\mu g\,mL^{-1}$ against the bacterial strains examined. If DNA gyrase was psammaplin A's primary antibacterial target, one would not expect a 10-fold discrepancy between these values.[14]

In order to resolve this issue, we sought to more accurately measure psammaplin A's inhibitory effect on bacterial DNA gyrase by conducting the assay in the absence of the dithiothreitol, thus, avoiding in situ reduction of the disulfide moiety. Suprisingly, and as shown in Figure 4, under these

Figure 4. DNA gyrase assay. Lane A: relaxed plasmid pBR322; B: supercoiled plasmid pBR322; C: relaxed pBR322+DNA gyrase; D–O: relaxed pBR322+DNA gyrase+compound at indicated concentration.

more stringent assay conditions, psammaplin A exhibited no detectable inhibition of bacterial DNA gyrase up to 100 $\mu g\,mL^{-1}$, while at the same time ciprofloxacin, a known DNA gyrase inhibitor used as a control, exhibited activity at concentrations well below 10 $\mu g\,mL^{-1}$. Moreover, analogue **31** (see structure in Table 1) with an average MIC of 1.13 $\mu g\,mL^{-1}$ against SA and MRSA also exhibited no detectable inhibitory activity against the DNA gyrase up to 100 $\mu g\,mL^{-1}$ as illustrated in Figure 4. These data suggest that psammaplin A, and hence its heterodimeric analogues, do not inhibit DNA gyrase activity to a significant extent. Perhaps the weak inhibitory activity observed by the earlier authors[9] could be attributed to the presence of the free thiol rather than the natural product itself, leaving the agent's primary target still to be defined.[15]

In an attempt to foster additional studies aimed at identification of such a target, we considered all the SAR data garnered during our program for hints as to the plausible mode of action of these compounds. Perhaps the most pertinent SAR observation from a mechanistic perspective was that the disulfide moiety is essential for potent biological activity. This finding suggested that psammaplin A and its analogues may be potentially operating through one of two precedented mechanisms.[16] A first potential mechanism, common for disulfide-containing molecules, is to act in a non-specific way to deplete cellular glutathione and/or cysteine, enabling subsequent redox cycling which generates free radicals capable of damaging the cellular machinery. It is through such a mechanism in large part that disulfides, such as phenyl disulfide and 4,4′-diaminophenyl disulfide, exert their cytotoxic effects.[17] A second potential mechanism of action for disulfide-containing molecules involves a specific interaction of the molecule with a particular cellular target, leading to covalent modification of the biological target via nucleophilic attack of a cysteine thiol on the electrophilic disulfide unit, resulting in the formation, of a mixed disulfide. As an example, the secondary fungal metabolite gliotoxin (**91**, Figure 5), which contains a disulfide bridge, has been shown to act through such a mechanism to inactivate a number of enzymatic targets.[18]

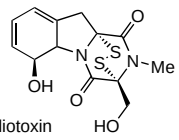

Figure 5. Molecular structure of the disulfide-containing natural product gliotoxin.

In the absence of definitive evidence of the molecular target of this series of antibacterial agents at the present time, we can only speculate as to through which type of mechanism these compounds act, based on our SAR observations. Nonetheless, such a prediction serves an important role from both the medicinal and biochemical points of views. First, these compounds are only of significant medicinal value if, in fact, they act at a specific molecular target. Second, it is prudent that before embarking on biochemical efforts to identify a specific molecular target, one should be reasonably assured that this class of compounds do not function through non-specific mechanisms.

At least three pieces of evidence, derived from our SAR data, point to action on a specific target(s) rather than a non-specific, redox cycling mechanism for the psammaplin A family of natural products. First, if a non-specific mechanism was responsible for the antibacterial properties of psammaplin A and its analogues, one would expect that structural modifications near the disulfide moiety would have a significant effect, while modifications at distant sites would not significantly alter the potency, yet this is not the case. For example, alkylation of the oxime functionality (i.e., **53** → **71**, Table 5) resulted in a 15-fold loss of activity, while moving of a

bromine substituent from the 3-position to the 4-position of the aryl ring in the "left" sububit (i.e., **57** → **59**, Table 2) resulted in a 50-fold loss of activity. The fact that these changes, which alter neither the steric nor the electronic environment of the disulfide unit, can have such a dramatic effect on the biological activity of these compounds argues strongly against the non-specific mechanism.

A second argument against a redox cycling mechanism is based on the fact that the introduction of steric congestion (in the form of 2′,6′-dimethyl substitution on the "right" hand aryl ring) around the disulfide linkage translated into highly potent compounds. For example, heterodimer **70** (Table 5) has an average MIC value of 0.12 μg mL^{-1}. If the cytotoxicity of this class of compounds resulted from a non-specific nucleophilic attack followed by oxidative recombination, one would expect a compound such as **70** (wherein both random nucleophic attack and recombination are hindered) to be quite inactive, which is not the case.

A final illuminating piece of evidence, which argues against the non-specific mechanism, is the general potency of this class of compounds. For example, known redox-cyclers such as phenyl disulfide and 4,4′-diaminodiphenyldisulfide were found to be inactive at 50 μg mL^{-1} against the bacterial strains used in this study,[1] while our best heterodimeric analogues are active well below 1 μg mL^{-1}.

While in the absence of a defined molecular target, we cannot categorically rule out the possibility of some non-specific toxicity, it seems likely that there exists one or more target(s) for psammaplin A and its analogues. Potentially, these compounds may bind to an enzyme (or family of enzymes) through non-covalent interactions as illustrated in Figure 6. Conceivably, a pseudo-intramolecular attack might

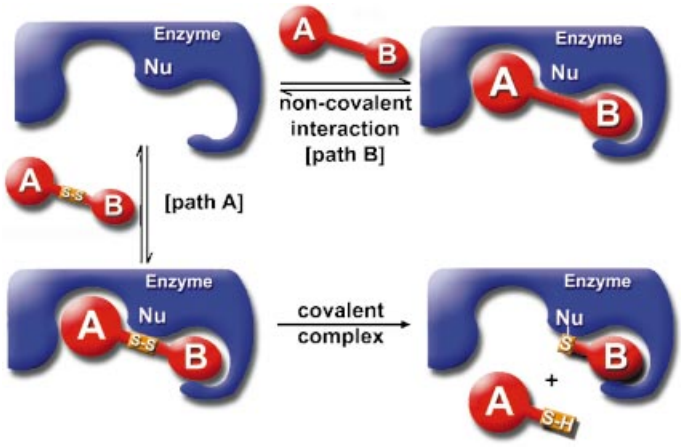

Figure 6. Proposed mechanism of action of psammaplin A-type antibacterial agents based on observed structure activity relationships.

then occur from a nearby nucleophile (such as a cysteine residue) onto the electrophilic disulfide moiety, leading to a covalent modification and deactivation of the enzyme (see path *A*, Figure 6). This proposal also offers an explanation for why compounds such as **82**, **84**, and **87** (Table 7), wherein the disulfide unit has been replaced with a structurally equivalent linkage, still retain low level antibacterial activity. One could envision, that since they have a similar overall structure to their disulfide-containing counterparts, these non-disulfides could act as non-covalent inhibitors of the same molecular target(s) as illustrated in path B of Figure 6. Based on the therapeutic potential of these compounds, we suggest that further biochemical investigations aimed at identifying their biological target(s) are warranted.

Conclusion

In this and the preceding paper[1] we have described the construction of a combinatorial library modeled after the marine natural product psammaplin A (**1**) whose antibacterial properties and symmetrical disulfide-based structure provided the impetus for this investigation. Antibacterial screening of this library led to the identification of a collection of simple, yet highly potent analogues whose structures were subsequently refined by high-throughput structure – activity studies. These efforts culminated in the discovery of several optimized antibacterial agents, some possessing more than 50-fold higher activities (e.g. **57**, **58**, and **70**, Figure 2) than the natural product. Most significantly, a number of these agents exhibited increased selectivity against bacterial over fibroblast and lymphocyte cells as compared to the natural product. In addition, these studies provided insight into the mechanism by which these compounds may act. Specifically, these investigations failed to confirm the previously reported DNA gyrase inhibition by psammaplin A. However, our SAR studies also argue against a non-specific redox-based mechanism of action for these compounds, suggesting that there exists an as of yet unidentified, biological target upon which this class of antibacterial agents act. Studies aimed at identifying such a target using modified versions of the described lead compounds are currently underway and will be reported in due course.

Experimental Section

General: See the preceding article[1] for general techniques employed and the preparation of compounds **2**, **3**, **4**, **5**, **20**, **21**, **22**, **30**, and **31**.

Synthesis of homodimeric disulfide 14: Diethyl acetimidomalonate (21.1 g, 1.0 equiv, 100 mmol) was added to a solution of NaOEt (6.8 g, 1.0 equiv, 100 mmol) in EtOH (80 mL). After 10 min, 3,5-difluorobenzyl bromide (20.7 g, 1.0 equiv, 100 mmol) was added and the reaction mixture was heated to reflux. After 12 h, H_2O (80 mL) was added and the reaction mixture was slowly cooled to 23 °C. The white precipitate that formed was collected by filtration and washed with Et_2O (10 mL) and H_2O (10 mL) to provide crude intermediate **7** which was dissolved in 48 % aq. HBr (70 mL) and the reaction mixture was heated to reflux. After 4 h, the reaction mixture was cooled to 23 °C, and the resultant precipitate was isolated by filtration. This solid was dissolved in warm H_2O and decolorized with activated carbon. After removal of the activated carbon, by filtration, Et_3N was added to bring the pH of the solution to 6.0. The white precipitate (**8**) formed on standing was isolated by filtration. From this point, compound **8** was converted to homodimer **14** in a manner identical to that described for the synthesis of psammaplin A.[1]

Compound 14: $R_f = 0.33$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3397, 2871, 1663, 1624, 1595, 1527, 1457, 1314, 1202, 1117, 1017, 846, 715 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.03$ (tt, $J = 7.3$, 7.3 Hz, 1H), 6.8 (dm, $J = 6.2$ Hz, 2H), 3.8 (s, 2H), 3.4 (q, $J = 6.7$ Hz, 2H), 2.8 (t, $J =$ 6.7 Hz, 2H); ^{13}C NMR (125 MHz, [D_6]DMSO): $\delta = 163.1$, 161.1, 150.7,

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141.3, 111.9, 11.6, 101.7, 38.2, 36.9, 28.8; HRMS (MALDI-FTMS): calcd for $C_{22}H_{22}F_4N_4O_4S_2$ $[M+H]^+$: 547.1091, found: 547.1088.

Synthesis of heterodimeric disulfide 17: Dithiothreitol (1.0 mg, 0.15 equiv, 0.006 mmol) was added at 23 °C to a solution of homodimer **14** (20.0 mg, 1.0 equiv, 0.04 mmol) and homodimer **15** (9.3 mg, 1.0 equiv, 0.04 mmol) in THF/phosphate buffer (pH 8.3) 3:1 (1.5 mL). After stirring for 12 h, the THF was evaporated from the reaction mixture and the aqueous phase was extracted with EtOAc (10 mL). The organic layer was washed with 10% aq. HCl (5 mL), dried over Na_2SO_4, and evaporated to dryness. The resulting residue was purified by preparative thin-layer chromatography to yield heterodimer **17** (5.44 mg, 34%). $R_f = 0.56$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3406$, 1660, 1619, 1595, 1527, 1454, 1302, 1225, 1114, 1002 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.55$ (m, 2H), 7.06 (t, $J = 8.8$ Hz, 2H), 6.85 (d, $J = 8.2$ Hz, 2H), 6.69–6.74 (m, 1H), 3.88 (br s, 2H), 3.53 (t, $J = 6.7$ Hz, 2H), 2.86 (t, $J = 6.8$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 167.6$ (dd, $J = 260.0$, 7.5 Hz), 165.8, 160.8, 160.1 (d, $J = 260.0$ Hz), 144.6 (d, $J = 2.5$ Hz), 142.8 (t, $J = 7.5$ Hz), 143.6, 141.2, 130.5, 116.3 (d, $J = 21.9$ Hz), 111.8 (d, $J = 20.0$ Hz), 101.7 (t, $J = 24.8$ Hz), 37.9, 36.8, 28.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{15}F_3N_2O_2S_2$ $[M+H]^+$: 401.0600, found: 401.0608.

Compounds **18**–**35**, **42**–**72**, and **88** were prepared in 18–42% yield by scrambling the appropriate homodimers as described above for the construction of heterodimer **17** from homodimers **14** and **15**.

Compound 18: $R_f = 0.25$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3402$, 1656, 1498, 1428, 1288, 1019 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.27$ (d, $J = 1.6$ Hz, 1H), 7.12–7.08 (m, 2H), 6.99 (t, $J = 1.6$ Hz, 1H), 6.88 (dq, $J = 6.1$, 0.6 Hz, 1H), 6.85 (d, $J = 6.7$ Hz, 1H), 6.65 (ddd, $J = 6.4$, 1.6, 0.5 Hz, 1H), 3.85 (s, 2H), 3.59 (t, $J = 5.4$ Hz, 2H), 2.92 (t, $J = 5.4$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 164.8$, 152.0, 151.6, 148.8, 137.7, 130.4, 129.7, 129.3, 128.7, 120.3, 117.2, 116.4, 114.4, 114.2, 38.3, 27.7; HRMS (MALDI-FTMS): calcd for $C_{17}H_{18}ClN_3O_3S_2$ $[M+H]^+$: 412.0551, found: 412.0562.

Compound 19: $R_f = 0.26$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3381$, 1661, 1513, 1431, 1362, 1292, 1223, 1019 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.30$ (dd, $J = 7.6$, 1.5 Hz, 1H), 7.10 (d, $J = 2.4$ Hz, 1H), 6.99 (td, $J = 5.6$, 1.5 Hz, 1H), 6.93 (dd, $J = 8.5$ Hz, 2.3 Hz, 1H), 6.68–6.64 (m, 2H), 6.49 (td, $J = 7.6$, 1.5 Hz, 1H), 3.68 (s, 2H), 3.49 (t, $J = 6.4$ Hz, 2H), 2.72 (t, $J = 7.4$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 164.8$, 153.2, 152.8, 150.5, 136.7, 132.2, 131.6, 130.4, 129.8, 119.0, 117.5, 116.9, 39.6, 37.7, 28.9.

Compound 24: $R_f = 0.77$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3406$, 1658, 1514, 1428, 1289, 1219, 1016, 712 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.40$ (d, $J = 8.8$ Hz, 2H), 7.21 (d, $J = 8.8$ Hz, 2H), 7.09 (d, $J = 2.0$ Hz, 1H), 6.95 (dd, $J = 8.8$, 2.0 Hz, 1H), 6.66 (d, $J = 8.5$ Hz, 1H), 3.67 (s, 2H), 3.41 (t, $J = 6.7$ Hz, 2H), 2.78 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 163.7$, 152.3, 151.8, 136.1, 132.3, 130.3, 130.0, 129.7, 129.4, 128.8, 116.9, 114.8, 38.4, 37.5, 28.3; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}Cl_2N_2O_3S_2$ $[M+Na]^+$: 452.9877, found: 452.9877.

Compound 25: $R_f = 0.44$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3218$, 1653, 1511, 1490, 1425, 1287, 1221, 1015 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.44$ (m, 2H), 7.09 (d, $J = 2.1$ Hz, 1H), 6.98–6.90 (m, 3H), 6.66 (d, $J = 8.2$ Hz, 1H), 3.67 (s, 2H), 3.42 (t, $J = 6.7$ Hz, 2H), 2.75 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 165.8$, 160.6 (d, $J = 240$ Hz), 151.6, 144.7, 130.6, 129.4, 127.6, 127.3, 120.1, 116.8, 115.9, 37.9, 36.9, 28.5; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}ClFN_2O_3S_2$ $[M+H]^+$: 415.0275, found: 415.0333.

Compound 26: $R_f = 0.28$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3400$, 1660, 1495, 1430, 1288, 1245, 1023, 825 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.36$ (d, $J = 8.5$ Hz, 2H), 7.09 (d, $J = 2.5$ Hz, 1H), 6.91 (dd, $J = 8.5$, 2.1 Hz, 1H), 6.78 (d, $J = 8.6$ Hz, 2H), 6.60 (d, $J = 8.6$ Hz, 1H), 3.67 (s, 3H), 3.67 (s, 2H), 3.43 (t, $J = 7.0$ Hz, 2H), 2.72 (dd, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 163.2$, 159.7, 151.7, 151.1, 132.1.129.8, 128.4, 126.9, 119.1, 116.4, 115.0, 114.9, 55.3, 36.9, 27.7; HRMS (MALDI-FTMS): calcd for $C_{18}H_{19}ClN_2O_4S_2$ $[M+Na]^+$: 449.0372, found: 449.0368.

Compound 27: $R_f = 0.29$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3384$, 1662, 1512, 1431, 1362, 1276, 1227, 1020, 951, 827, 708 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.30$ (d, $J = 6.7$ Hz, 2H), 7.09 (s, 1H), 6.91 (m, 1H), 6.68–6.64 (m, 3H), 3.68 (s, 2H), 3.45 (t, $J = 7.1$ Hz, 2H), 2.71 (d, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 165.9$, 160.8, 156.5, 151.7, 141.6, 131.8, 130.6, 127.6, 127.1, 119.4, 116.2, 115.9, 40.9, 38.6, 30.1; HRMS (MALDI-FTMS): calcd for $C_{17}H_{17}ClN_2O_4S_2$ $[M+Na]^+$: 435.0216, found: 435.0223.

Compound 28: $R_f = 0.54$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3396$, 1660, 1425, 1202, 1127, 1021, 882 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.56$ (d, $J = 2.0$ Hz, 1H), 7.34–7.33 (m, 2H), 7.08 (d, $J = 2.0$ Hz, 1H), 6.92 (dd, $J = 8.5$, 2.1 Hz, 1H), 6.65 (d, $J = 8.2$ Hz, 1H), 3.67 (s, 2H), 3.42 (t, $J = 6.7$ Hz, 2H), 2.79 (t, $J = 6.4$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 165.8$, 160.8, 151.7, 142.4, 136.3, 134.1, 133.8, 130.6, 130.1, 128.4, 127.6, 127.1, 119.4, 115.9, 40.9, 38.6, 30.1; HRMS (MALDI-FTMS): calcd for $C_{17}H_{15}Cl_3N_2O_3S_2$ $[M+Na]^+$: 486.9487, found: 486.9484.

Compound 29: $R_f = 0.29$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3418$, 1649, 1513, 1425, 1290, 1213, 1019, 672 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.25$ (d, $J = 8.5$ Hz, 1H), 7.10 (d, $J = 2.4$ Hz, 1H), 6.95 (dd, $J = 8.8$, 2.1 Hz, 1H), 6.68–6.65 (m, 2H), 6.44 (dd, $J = 8.2$, 2.4 Hz, 1H), 3.68 (s, 2H), 3.49 (t, $J = 7.0$ Hz, 2H), 2.72 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 162.3$, 150.9, 150.4, 149.9, 135.9, 134.5, 128.9, 127.5, 118.2, 115.5, 114.7, 114.3, 112.8, 39.5, 35.1, 26.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{17}Cl_2N_3O_3S_2$ $[M+H]^+$: 446.0088, found: 446.0170.

Compound 32: $R_f = 0.38$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3413$, 1657, 1526, 1488, 1418, 1287, 1221, 1018 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.59$ (d, $J = 1.7$ Hz, 1H), 7.53 (q, $J = 5.3$ Hz, 1H), 7.09–7.03 (m, 2H), 6.68 (d, $J = 8.2$ Hz, 1H), 3.75 (s, 2H), 3.52 (t, $J = 6.7$ Hz, 2H), 2.85 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 165.8$, 160.8, 160.6 (d, $J = 260$ Hz), 155.3, 138.7, 130.5, 130.0, 129.4, 116.5, 116.3, 114.7, 85.6, 39.1, 36.9, 27.4; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}FIN_2O_3S_2$ $[M+H]^+$: 506.9631, found: 506.9704.

Compound 33: $R_f = 0.49$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3318$, 2926, 1654, 1463, 1438, 1382, 1221, 1076, 1020 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.58$ (d, $J = 1.7$ Hz, 1H), 7.49 (d, $J = 8.5$ Hz, 2H), 7.30 (d, $J = 8.5$ Hz, 2H), 7.08 (dd, $J = 8.5$, 2.1 Hz, 1H), 6.68 (d, $J = 8.5$ Hz, 1H), 3.75 (s, 2H), 3.53 (t, $J = 6.5$ Hz, 2H), 2.86 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 165.8$, 160.8, 155.3, 146.2, 136.8, 132.8, 131.8, 130.5, 129.1, 128.9, 116.6, 85.6, 40.9, 38.6, 30.5; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}ClIN_2O_3S_2$ $[M+H]^+$: 522.9336, found: 522.9415.

Compound 34: $R_f = 0.35$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3403$, 1657, 1528, 1487, 1451, 1222, 1153, 1020 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.55$ (q, $J = 5.3$ Hz, 1H), 7.08–6.99 (m, 4H), 6.70 (br s, 1H), 6.57 (dd, $J = 8.8$, 1.8 Hz, 1H), 3.82 (s, 2H), 3.52 (t, $J = 6.7$ Hz, 2H), 2.85 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 163.8$, 160.6 (d, $J = 260$ Hz), 157.7, 152.2, 138.5, 131.4, 131.0, 129.6, 119.9, 116.1, 115.2, 113.5, 38.5, 37.3, 29.3; HRMS (MALDI-FTMS): calcd for $C_{17}H_{17}FN_2O_3S_2$ $[M+H]^+$: 381.0665, found: 381.0733.

Compound 35: $R_f = 0.77$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3266$, 1661, 1525, 1472, 1278, 1014, 777, 450 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.49$ (d, $J = 8.5$ Hz, 2H), 7.30 (d, $J = 8.5$ Hz, 2H), 7.01 (t, $J = 8.2$ Hz, 1H), 6.70 (br s, 1H), 6.56 (dd, $J = 9.0$, 2.3 Hz, 1H), 3.81 (s, 2H), 3.50 (t, $J = 6.7$ Hz, 2H), 2.85 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 163.3$, 157.2, 152.3, 137.9, 135.6, 134.5, 132.5, 131.7, 129.5, 129.2, 128.9, 119.4, 115.6, 113.0, 38.2, 36.9, 28.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{17}ClN_2O_3S_2$ $[M+H]^+$: 397.0369, found: 397.0431.

Compound 42: $R_f = 0.66$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3392$, 1661, 1524, 1488, 1221, 1019, 826, 704 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.48–7.46$ (m, 2H), 7.16–7.11 (m, 4H), 7.01–6.96 (m, 3H), 3.81 (s, 1H), 3.44–3.40 (m, 2H), 2.77–2.75 (m, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 163.0$, 160.8, 160.6 (d, $J = 260$ Hz), 151.4, 136.4, 130.5, 130.2, 128.4, 127.9, 125.7, 116.4, 115.9, 37.7, 36.6, 28.6; HRMS (MALDI-FTMS): calcd for $C_{17}H_{17}FN_2O_2S_2$ $[M+Na]^+$: 387.0613, found: 387.0609.

Compound 43: $R_f = 0.28$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3354$, 1660, 1590, 1525, 1490, 1448, 1290 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.21–7.10$ (m, 7H), 6.55–6.53 (m, 2H), 3.80 (s, 2H), 3.47–3.43 (m, 2H), 2.70 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 165.8$, 160.8, 143.9, 142.1, 138.5, 130.0, 129.3, 127.2, 127.2, 125.6, 119.3, 40.9, 38.6, 29.9; HRMS (MALDI-FTMS): calcd for $C_{17}H_{19}N_3O_2S_2$ $[M+Na]^+$: 384.0816, found: 384.0820.

Compound 44: $R_f = 0.28$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} = 3402$, 1661, 1525, 1492, 1291, 1227, 1022 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.30$ (d, $J = 8.5$ Hz, 2H), 7.18–7.10 (m, 1H), 6.63 (d, $J = 8.5$ Hz, 2H), 3.92 (s, 2H), 3.55 (t, $J = 6.7$ Hz, 2H), 2.80 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 163.7$, 161.6, 159.6, 151.4, 150.1, 134.8, 130.6, 128.6, 124.0, 120.1, 115.5, 114.6, 38.7, 36.6, 22.6; HRMS

(MALDI-FTMS): calcd for $C_{17}H_{18}FN_3O_2S_2$ $[M+Na]^+$: 402.0722, found: 402.0720.

Compound 45: $R_f = 0.65$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3419, 1656, 1527, 1488, 1226, 1018, 757 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.58-7.54$ (m, 2H), 7.19–6.97 (m, 6H), 3.91 (s, 2H), 3.52 (t, $J = 6.7$ Hz, 2H), 2.86 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.5$, 161.5 (d, $J = 260$ Hz), 160.6 (d, $J = 260$ Hz), 158.7 (d, $J = 2.5$ Hz), 144.6 (d, $J = 2.5$ Hz), 123.9 (d, $J = 2.5$ Hz), 122.9 (d, $J = 28.7$ Hz), 116.2 (d, $J =$ 27.5 Hz), 115.7 (d, $J = 27.5$ Hz), 38.7, 37.4, 22.6; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}F_2N_2O_2S_2$ $[M+Na]^+$: 405.0518, found: 405.0513.

Compound 46: $R_f = 0.37$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3388, 1661, 1524, 1289, 1021 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.19$ (d, $J = 8.2$ Hz, 1H), 7.13–7.12 (m, 1H), 6.97 (d, $J = 7.6$ Hz, 1H), 6.89 (d, $J =$ 10.2 Hz, 1H), 6.78–6.76 (m, 1H), 6.53 (d, $J = 8.2$ Hz, 1H), 3.80 (s, 2H), 3.47–3.44 (m, 2H), 2.70 (t, $J = 6.8$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.3$ (d, $J = 2.8$ Hz), 160.9 (d, $J = 239$ Hz), 151.8, 150.1, 134.5, 133.1 (d, $J = 2.8$ Hz), 130.7 (d, $J = 2.8$ Hz), 120.9, 119.9, 115.1 (d, $J =$ 28.5 Hz), 114.3 (d, $J = 27.3$ Hz), 38.2, 36.3, 28.4; HRMS (MALDI-FTMS): calcd for $C_{17}H_{18}FN_3O_2S_2$ $[M+H]^+$: 379.0824, found: 379.0833.

Compound 47: $R_f = 0.57$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3392, 2882, 1664, 1581, 1510, 1443, 135, 1197, 1136, 1017, 949, 813, 778, 714 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.30$ (d, $J = 8.5$ Hz, 2H), 7.11 (q, $J = 1.8$ Hz, 1H), 6.97 (d, $J = 7.6$ Hz, 1H), 6.90 (d, $J = 10.0$ Hz, 1H), 6.78–6.76 (m, 1H), 6.58 (d, $J = 8.8$ Hz, 2H), 3.80 (s, 2H), 3.46 (t, $J = 6.8$ Hz, 2H), 2.84 (s, 6H), 2.71 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta =$ 163.2, 151.2, 150.7, 139.6, 133.8, 130.2, 124.8, 120.5, 115.5, 115.3, 112.6, 40.4, 38.1, 36.2, 28.7; HRMS (MALDI-FTMS): calcd for $C_{19}H_{22}FN_3O_2S_2$ $[M+H]^+$: 408.1137, found: 408.1208.

Compound 48: $R_f = 0.76$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.67$ (td, $J = 7.6$, 1.5 Hz, 1H), 7.29–7.03 (m, 5H), 6.97 (d, $J = 10.3$ Hz, 1H), 6.85 (td, $J = 8.8$, 2.3 Hz, 1H), 3.87 (s, 2H), 3.53 (t, $J = 6.7$ Hz, 2H), 2.86 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.2$, 162.7 (d, $J = 260$ Hz), 161.3 (d, $J = 260$ Hz), 160.8, 140.8, 138.4, 131.2, 130.6, 130.2, 125.3, 124.8, 116.8 (d, $J = 25.0$ Hz), 115.5 (d, $J = 25$ Hz), 115.1, 37.9, 37.0, 28.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}F_2N_2O_2S_2$ $[M+H]^+$: 383.0621, found: 383.0706.

Compound 49: $R_f = 0.78$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3400, 1665, 1588, 1524, 1486, 1441, 1223, 1018 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.46-7.43$ (m, 2H), 7.14–7.09 (m, 1H), 6.98–6.93 (m, 3H), 6.89 (dd, $J = 10.3$, 1.7 Hz, 1H), 6.79 (td, $J = 8.8$, 2.6 Hz, 1H), 3.80 (s, 2H), 3.43 (t, $J = 6.7$ Hz, 2H), 2.75 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.3$, 162.1 (d, $J = 240$ Hz), 161.2 (d, $J = 240$ Hz), 151.3 (d, $J = 2.8$ Hz), 139.6 (d, $J = 7.6$ Hz), 132.2 (d, $J = 2.8$ Hz), 130.9 (d, $J =$ 8.5 Hz), 130.6 (d, $J = 8.5$ Hz), 130.2 (d, $J = 8.5$ Hz), 124.9 (d, $J = 2.8$ Hz), 116.4 (d, $J = 22.0$ Hz), 115.4 (d, $J = 21.0$ Hz), 113.0 (d, $J = 21.0$ Hz), 38.0, 36.9, 28.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}F_2N_2O_2S_2$ $[M+Na]^+$: 405.0518, found: 405.0530.

Compound 50: $R_f = 0.63$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3398, 1664, 1510, 1472, 1431, 1220, 1091, 1014, 818, 736, 490 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.41-7.38$ (m, 2H), 7.22–7.18 (m, 2H), 7.14–7.08 (m, 1H), 6.97 (dq, $J = 7.6$, 0.8 Hz, 1H), 6.89 (dq, $J = 11.7$, 1.8 Hz, 1H), 6.78 (tdd, $J = 9.1$, 2.6, 0.9 Hz, 1H), 3.80 (s, 2H), 6.97 (t, $J = 6.7$ Hz, 2H), 2.76 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.6$, 162.5 (d, $J =$ 240 Hz), 151.5, 139.9, 135.1, 130.4 (d, $J = 9.5$ Hz), 130.1, 129.4, 128.5 (d, $J =$ 14 Hz), 127.6, 125.1 (d, $J = 2.8$ Hz), 115.7 (d, $J = 22$ Hz), 113.2 (d, $J =$ 20 Hz), 38.3, 37.2, 29.1; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}ClFN_2O_2S_2$ $[M+H]^+$: 399.0326, found: 399.0413.

Compound 51: $R_f = 0.65$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3404, 2872, 1663, 1583, 1524, 1486, 1463, 1245, 1207, 1022, 774 cm^{-1}, ^1H NMR (400 MHz, CD_3OD): $\delta = 7.41-7.38$ (m, 2H), 7.22 -7.18 (m, 2H), 7.16–7.08 (m, 1H), 6.97 (dq, $J = 7.6$, 0.8 Hz, 1H), 6.89 (dq, $J = 11.7$, 1.8 Hz, 1H), 6.78–6.77 (m, 1H), 3.80 (s, 2H), 3.42 (t, $J = 6.7$ Hz, 2H), 2.76 (t, $J =$ 6.7 Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.1$, 162.2 (d, $J =$ 240 Hz), 160.1, 150.3, 138.6 (d, $J = 8.1$ Hz), 138.2, 132.9, 132.0 (d, $J =$ 7.5 Hz), 129.8, 125.2, 123.9, 114.4 (d, $J = 20.9$ Hz), 111.9 (d, $J = 20.0$ Hz), 37.0, 35.9, 27.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}ClFN_2O_3S_2$ $[M+H]^+$: 399.0326, found: 399.0413.

Compound 52: $R_f = 0.69$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3415, 1663, 1523, 1477, 1441, 1244, 1207, 1022, 821, 773 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.21-7.12$ (m, 6H), 6.55–6.53 (m, 2H), 3.80 (s, 2H), 3.47–3.42 (m, 2H), 2.72–2.68 (m, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 162.2$, 161.0 (d, $J = 240$ Hz), 150.3 (d, $J = 28.6$ Hz), 138.6, 134.7, 130.8, 129.1 (d, $J = 8.5$ Hz), 128.3, 127.9, 123.9 (d, $J = 1.9$ Hz), 114.4 (d, $J = 20.9$ Hz), 111.9 (d, $J = 20.9$ Hz), 37.2, 36.1, 27.8; MS (ESI): calcd for $C_{17}H_{16}ClFN_2O_2S_2$ $[M+H]^+$: 399, found: 399.

Compound 53: $R_f = 0.66$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3388, 1665, 1512, 1432, 1221, 1017, 825 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.46-7.41$ (m, 2H), 7.18–7.13 (m, 2H), 6.98–6.92 (m, 2H), 6.85–6.79 (m, 2H), 3.75 (s, 2H), 3.43–3.39 (m, 2H), 2.76–2.72 (m, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.0$ (d, $J = 3.8$ Hz), 161.8 (d, $J = 244.1$ Hz), 161.3 (d, $J = 246.3$ Hz), 159.4, 151.3 (d, $J = 38.1$ Hz), 132.6 (d, $J = 3.8$ Hz), 130.5 (d, $J = 7.3$ Hz), 130.3 (d, $J = 8.5$ Hz), 116.1 (d, $J = 21.0$ Hz), 114.7 (d, $J = 20.9$ Hz), 37.6, 36.6, 27.9; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}F_2N_2O_2S_2$ $[M+Na]^+$: 405.0518, found: 405.0513.

Compound 54: $R_f = 0.51$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3210, 1663, 1424, 1488, 1221, 1015, 828 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.40$ (d, $J = 8.5$ Hz, 2H), 7.21 (d, $J = 8.8$ Hz, 2H), 7.16 (q, $J = 5.6$ Hz, 2H), 6.83 (t, $J = 9.1$ Hz, 2H), 3.76 (s, 2H), 3.41 (t, $J = 6.7$ Hz, 2H), 2.76 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.3$ (d, $J =$ 5.7 Hz), 160.7 (d, $J = 240$ Hz), 151.7 (d, $J = 8.5$ Hz), 137.7, 132.9 (d, $J =$ 2.8 Hz), 130.6 (d, $J = 7.6$ Hz), 129.5, 129.2, 128.9 (d, $J = 2.8$ Hz), 115.0 (d, $J = 20.9$ Hz), 38.0, 37.1, 28.2 ; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}ClFN_2O_2S_2$ $[M+H]^+$: 399.0326, found: 399.0409.

Compound 55: $R_f = 0.36$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3281, 1660, 1619, 1595, 1526, 1463, 1307, 1202, 116, 1007 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.50$ (dm, $J = 8.5$ Hz, 2H), 7.31 (dm, $J = 8.8$ Hz, 2H), 6.85 (dm, $J = 8.2$ Hz, 2H), 6.72 (tm, $J = 9.4$ Hz, 1H), 3.88 (s, 2H), 3.53 (t, $J = 6.4$ Hz, 2H), 2.88 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.4$, 162.0 (dd, $J = 282.1$, 9.5 Hz), 150.7, 141.2 (t, $J =$ 6.2 Hz), 135.6, 131.8, 129.2, 128.9, 111.7 (d, $J = 30.3$ Hz), 101.7 (t, $J =$ 28.7 Hz), 132.9 (d, $J = 2.8$ Hz), 130.6 (d, $J = 7.6$ Hz), 129.5, 129.2, 128.9 (d, $J = 2.8$ Hz), 115.0 (d, $J = 20.9$ Hz), 38.0, 37.0, 28.8.

Compound 56: $R_f = 0.62$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3285, 1660, 1525, 1480, 1426, 1205, 1090, 1012, 811 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.40$ (d, $J = 8.5$ Hz, 2H), 7.21 (d, $J = 8.5$ Hz, 2H), 7.12 (m, 4H), 3.76 (s, 2H), 3.41 (t, $J = 6.7$ Hz, 2H), 2.77 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.3$, 151.4, 135.8, 135.7, 130.6, 129.5, 129.2, 128.9, 128.2, 38.0, 37.0, 28.4; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}Cl_2N_2O_2S_2$ $[M+H]^+$: 415.0030, found: 415.0094.

Compound 57: $R_f = 0.52$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3398, 1669, 1529, 1485, 1429, 1222, 1019 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.24$ (s, 1H), 7.12–7.09 (m, 2H), 7.04 (br d, $J = 7.6$ Hz, 1H), 6.97–6.92 (m, 2H), 6.44 (d, $J = 8.5$ Hz, 2H), 3.69 (s, 2H), 3.36 (t, $J = 6.7$ Hz, 2H), 2.61 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.2$, 151.2, 149.1, 139.6, 134.4, 131.3, 130.5, 129.0, 127.8, 121.5, 114.3, 114.2, 38.2, 36.9, 28.7; HRMS (MALDI-FTMS): calcd for $C_{17}H_{18}BrN_3O_2S_2$ $[M+H]^+$: 440.0024, found: 440.0104.

Compound 58: $R_f = 0.53$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.36$ (m, 2H), 7.24 (br s, 1H), 7.11 (br d, $J =$ 7.9 Hz, 1H), 7.04 (br d, $J = 7.7$ Hz, 1H), 6.95 (t, $J = 7.9$ Hz, 1H), 6.87 (t, $J = 8.8$ Hz, 2H), 3.68 (s, 2H), 3.34 (t, $J = 6.7$ Hz, 2H), 2.66 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 164.0$, 160.6 (d, $J = 260$ Hz), 152.0, 144.6, 140.4, 131.2, 130.4, 130.0, 129.3 (d, $J = 8.5$ Hz), 129.1, 122.3, 117.2 (d, $J = 21.9$ Hz), 40.9, 37.7, 29.5; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}BrFN_2O_2S_2$ $[M+Na]^+$: 464.918, found: 464.9713.

Compound 59: $R_f = 0.49$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.36$ (d, $J = 8.5$ Hz, 2H), 7.28 (dm, $J = 8.5$ Hz, 2H), 6.63 (d, $J = 8.5$ Hz, 2H), 3.85 (s, 2H), 3.55 (t, $J = 6.7$ Hz, 2H), 2.79 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 162.7$, 150.9, 148.1, 135.9, 134.0, 130.8, 130.6, 119.3, 118.8, 113.8, 37.8, 36.6, 28.1; HRMS (MALDI-FTMS): calcd for $C_{17}H_{18}BrN_3O_2S_2$ $[M+Na]^+$: 461.9916, found: 461.9931.

Compound 60: $R_f = 0.61$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.54$ (m, 2H), 7.35 (dm, $J = 8.5$ Hz, 2H), 7.17 (d, $J = 8.2$ Hz, 2H), 7.05 (tm, $J = 8.8$ Hz, 2H), 3.84 (s, 2H), 3.52 (t, $J = 6.4$ Hz, 2H), 2.84 (t, $J = 6.5$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.2$, 163.1, 151.3, 131.1, 131.0, 130.8, 130.6, 130.5, 130.4, 118.1, 116.7, 116.5,116.3, 38.1, 37.9, 36.8, 28.5; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}BrFN_2O_2S_2$ $[M+H]^+$: 442.9821, found: 442.9893.

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Compound 61: $R_f = 0.69$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3278, 1659, 1528, 1479, 1011, 812 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta =$ 7.49 (d, $J = 8.5$ Hz, 2H), 7.35 (d, $J = 8.5$ Hz, 2H), 7.30 (d, $J = 8.8$ Hz, 2H), 7.17 (d, $J = 8.2$ Hz, 2H), 3.84 (s, 2H), 3.50 (t, $J = 6.4$ Hz, 2H), 2.86 (t, $J =$ 6.7 Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.7$, 160.1, 152.2, 151.8, 132.6, 131.3, 131.0, 130.4, 128.8, 116.8, 38.4, 37.4, 28.3; HRMS (MALDI-FTMS): calcd for $C_{17}H_{16}BrClN_2O_2S_2$ $[M+H]^+$: 458.9525, found: 458.9599.

Compound 62: $R_f = 0.39$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.91$ (s, 1H), 7.73–7.68 (m, 2H), 7.50 (dd, $J = 8.5$, 1.7 Hz, 1H), 7.39–7.35 (m, 2H), 7.08 (d, $J = 2.1$ Hz, 1H), 6.90 (dd, $J = 8.5$, 2.3 Hz, 1H), 6.64 (d, $J = 8.2$ Hz, 2H), 3.63 (s, 2H), 3.45 (t, $J = 6.7$ Hz, 2H), 2.81 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 160.8$, 150.6, 142.3, 132.8, 131.5, 130.9, 129.7, 128.5, 125.3, 125.0, 124.2, 124.0, 123.0, 120.9, 120.8, 120.0, 40.9, 38.6, 30.3; HRMS (MALDI-FTMS): calcd for $C_{21}H_{19}ClN_2O_3S_2$ $[M+H]^+$: 447.0526, found: 447.0614.

Compound 63: $R_f = 0.41$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3392, 1664, 1523, 1442, 1225, 1133, 1018, 949, 886, 779, 737 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.24$ (d, $J = 2.0$ Hz, 1H), 7.13 (m, 1H), 6.98 (dm, $J = 7.6$ Hz, 1H), 6.90 (dt, $J = 10.2$, 2.1 Hz, 1H), 6.78 (td, $J = 8.5$, 2.6 Hz, 1H), 6.37 (d, $J = 1.7$ Hz, 1H), 3.82 (s, 2H), 3.48 (t, $J = 6.7$ Hz, 2H), 2.73 (t, $J =$ 7.3 Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.2$, 162.1 (d, $J =$ 240 Hz), 151.2 (d, $J = 7.8$ Hz), 141.8, 139.5, 130.1, 124.8, 115.4 (d, $J =$ 20.9 Hz), 114.5, 112.9 (d, $J = 20.9$ Hz), 112.4, 38.0, 36.4, 28.7, 11.6; HRMS (MALDI-FTMS): calcd for $C_{16}H_{17}FN_2O_3S_2$ $[M+Na]^+$: 391.0562, found: 391.0560.

Compound 64: $R_f = 0.66$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 8.73$ (dd, $J = 4.4$, 1.7 Hz, 1H), 8.23 (dd, $J = 8.5$, 1.7 Hz, 1H), 8.06 (dd, $J = 7.6$, 0.88 Hz, 1H), 7.65 (dd, $J = 8.2$, 1.1 Hz, 1H), 7.48–7.43 (m, 2H), 7.16 (dd, $J = 8.5$, 5.3 Hz, 2H), 6.82 (t, $J = 6.7$ Hz, 2H), 3.75 (s, 2H), 3.45 (t, $J = 6.7$ Hz, 2H), 2.83 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 165.8$, 165.1 (d, $J = 260$ Hz), 160.8, 150.1, 147.9, 138.2, 136.4, 132.9, 131.5, 128.9, 128.8, 128.7, 124.4, 120.9, 113.7 (d, $J =$ 260 Hz), 41.0, 38.6, 29.9.

Compound 65: $R_f = 0.77$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.31$ (s, 1H), 7.12 (q, $J = 7.3$ Hz, 1H), 6.96 (d, $J = 7.0$ Hz, 1H), 6.88 (d, $J = 9.7$ Hz, 1H), 6.78–6.76 (m, 1H), 6.21–6.16 (m, 2H), 3.80 (s, 4H), 3.34 (t, $J = 6.4$ Hz, 2H), 2.44 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.2$, 162.1 (d, $J = 241$ Hz), 151.3, 150.1, 142.9, 139.6 (d, $J = 7.6$ Hz), 130.2 (d, $J = 7.6$ Hz), 124.9, 115.4 (d, $J = 20.9$ Hz), 113.0 (d, $J = 20.9$ Hz), 110.9, 109.1, 37.9, 36.8, 34.6, 28.8; HRMS (MALDI-FTMS): calcd for $C_{16}H_{17}FN_2O_3S_2$ $[M+Na]^+$: 391.0562, found: 391.0573.

Compound 66: $R_f = 0.68$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3403, 1663, 1523, 1442, 1211, 1023 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta =$ 7.43–7.41 (m, 1H), 7.19–7.18 (m, 1H), 7.14–7.10 (m, 1H), 6.97 (d, $J =$ 7.6 Hz, 1H), 6.88–6.86 (m, 2H), 6.79–6.74 (m, 1H), 3.80 (s, 2H), 3.49 (t, $J = 6.7$ Hz, 2H), 2.80 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.2$, 163.0, 162.0 (d, $J = 260$ Hz), 151.2, 139.6 (d, $J = 7.6$ Hz), 135.0 (d, $J = 8.5$ Hz), 132.4, 130.1 (d, $J = 8.6$ Hz), 128.2, 124.8, 115.4 (d, $J = 20.9$ Hz), 112.9 (d, $J = 20.9$ Hz), 37.8, 36.7, 28.7.

Compound 67: $R_f = 0.56$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3404, 1663, 1514, 1426, 1290, 1222, 1021, 777 cm^{-1};^1H NMR (400 MHz, CD_3OD): $\delta = 7.88–7.87$ (m, 1H), 7.38–7.36 (m, 2H), 7.24–7.21 (m, 1H), 7.10 (br s, 1H), 6.93–6.92 (m, 1H), 6.69–6.67 (m, 1H), 3.68 (s, 2H), 3.55–3.54 (m, 2H), 2.86–2.85 (m, 2H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta =$ 163.2, 150.8, 150.4, 138.9, 133.4, 131.3, 128.9, 128.4, 127.5, 127.4, 118.2, 115.4, 37.2, 36.3, 26.8; HRMS (MALDI-FTMS): calcd for $C_{17}H_{15}Cl_3N_2O_3S_2$ $[M+Na]^+$: 486.9487, found: 486.9491.

Compound 68: $R_f = 0.02$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3412, 1654, 1513, 1428, 1288, 1215, 1017, 685 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.10–7.09$ (m, 1H), 7.03–7.00 (m, 3H), 6.94–6.91 (m, 1H), 6.69–6.66 (m, 1H), 3.68 (s, 2H), 3.46–3.43 (m, 2H), 2.73–2.70 (m, 2H), 2.44 (s, 6H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.2$, 160.8, 151.5, 141.3, 138.4, 131.6, 130.6, 129.9, 128.5, 128.3, 119.5, 116.4, 38.5, 27.8, 21.7, 21.0; HRMS (MALDI-FTMS): calcd for $C_{19}H_{21}ClN_2O_3S_2$ $[M+Na]^+$: 447.0574, found: 447.0566.

Compound 69: $R_f = 0.67$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3390, 1666, 1527, 1457, 1362, 1314, 1201, 1019, 845, 775, 717 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 6.95–6.88$ (m, 3H), 6.66 (br d, $J = 6.4$ Hz, 2H), 6.53 (tm, $J = 9.1$ Hz, 1H), 3.70 (s, 2H), 3.36 (t, $J = 6.8$ Hz, 2H), 2.63 (t, $J = 6.8$ Hz, 2H), 2.34 (s, 6H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.1$, 162.2 (d, $J = 305.2$ Hz), 150.7, 142.7, 142.0, 141.3, 129.8, 128.3, 111.7 (d, $J =$ 305.2 Hz), 102.0 (t, $J = 30.3$ Hz), 36.9, 28.8, 21.7, 21.0; HRMS (MALDI-FTMS): calcd for $C_{19}H_{20}F_2N_2O_2S_2$ $[M+Na]^+$: 433.0832, found: 433.0891.

Compound 70: $R_f = 0.65$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3410, 1658, 1466, 1205, 1009 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.81$ (m, 1H), 7.23 (br s, 1H), 7.11 (dm, $J = 7.9$ Hz, 1H), 7.04 (dm, $J = 7.7$ Hz, 1H), 6.88–6.97 (m, 4H), 3.67 (s, 2H), 3.35 (q, $J = 6.2$ Hz, 2H), 2.62 (t, $J = 7.0$ Hz, 2H), 2.34 (s, 6H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta = 163.1$, 151.2, 142.0, 139.6, 131.3, 130.5, 129.4, 129.0, 128.5, 128.2, 127.8, 121.5, 36.8, 28.7, 21.7, 21.0; HRMS (MALDI-FTMS): calcd for $C_{19}H_{21}BrN_2O_2S_2$ $[M+H]^+$: 453.0228, found: 453.0286.

Compound 71: $R_f = 0.79$ (silica gel, EtOAc/hexanes 1:1); ^1H NMR (400 MHz, CD_3OD): $\delta = 7.43–7.40$ (m, 2H), 7.22–7.18 (m, 5H), 7.09–7.06 (m, 2H), 6.93–6.90 (m, 2H), 6.81–6.77 (m, 2H), 5.12 (s, 2H), 3.73 (m, 2H), 3.41 (t, $J = 7.0$ Hz, 2H), 2.74 (t, $J = 7.0$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 162.3$, 161.7 (d, $J = 243$ Hz), 160.7 (d, $J = 240$ Hz), 159.8, 152.3 (d, $J = 4.7$ Hz), 136.9 (d, $J = 1.9$ Hz), 132.1 (d, $J = 2.8$ Hz), 130.8 (d, $J = 8.5$ Hz), 130.6 (d, $J = 7.6$ Hz), 128.3 (d, $J = 3.8$ Hz), 128.0 (d, $J = 5.7$ Hz), 116.6 (d, $J = 21.9$ Hz), 116.3 (d, $J = 21.9$ Hz), 115.0 (d, $J = 20.9$ Hz), 76.4, 38.2, 36.7, 29.1; HRMS (MALDI-FTMS): calcd for $C_{24}H_{22}F_2N_2O_2S_2$ $[M+H]^+$: 473.1091, found: 473.1163.

Compound 72: $R_f = 0.72$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3411, 1670, 1611, 1431, 1223, 1046, 825 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.47–7.43$ (m, 2H), 7.13–7.10 (m, 2H), 6.98–6.94 (m, 2H), 6.84–6.81 (m, 2H), 3.90 (s, 3H), 3.76 (s, 2H), 3.42 (t, $J = 6.7$ Hz, 2H), 2.75 (t, $J =$ 6.4 Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 162.3$, 162.1 (d, $J =$ 243 Hz), 161.3 (d, $J = 240$ Hz), 159.8, 151.9, 132.1 (d, $J = 1.9$ Hz), 130.8 (d, $J = 8.5$ Hz), 130.6 (d, $J = 8.5$ Hz), 130.5 (d, $J = 8.5$ Hz), 116.6 (d, $J =$ 21.9 Hz), 116.3 (d, $J = 21.9$ Hz), 115.1 (d, $J = 20.9$ Hz), 62.6, 38.1, 36.6, 28.8; HRMS (MALDI-FTMS): calcd for $C_{18}H_{18}F_2N_2O_2S_2$ $[M+H]^+$: 397.0778, found: 397.0846.

Compound 88: $R_f = 0.72$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3388, 1662, 1513, 1430, 1287, 1226, 1018, 950 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.27–7.28$ (m, 6H), 7.10 (d, $J = 6.4$ Hz, 1H), 6.84 (dd, $J = 6.7$, 1.2 Hz, 2H), 6.80 (dd, $J = 5.6$, 1.2 Hz, 1H), 3.96 (s, 2H), 3.86 (s, 2H), 3.49 (t, $J = 5.3$ Hz, 2H), 2.58 (t, $J = 5.4$ Hz, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 163.2$, 158.1, 158.2, 151.8, 151.3, 151.2, 133.1, 133.0, 129.8, 128.5, 128.4, 116.3, 36.9, 36.5, 27.8; HRMS (MALDI-FTMS): calcd for $C_{18}H_{19}ClN_2O_3S_2$ $[M+Na]^+$: 433.0423, found: 433.0414.

Compound 73: This compound was prepared by a route similar to that used for the synthesis of psammaplin A[1] with the following two modifications: Bromotyrosine was replaced by chlorotyrosine and cystamine was replaced by 1,6-diaminohexane. **73**: $R_f = 0.11$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3403, 1658, 1511, 1430, 1289, 1219, 1022, 710 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 7.27$ (br s, 1H), 7.10 (d, $J = 6.4$ Hz, 1H), 6.84 (d, $J = 6.7$ Hz, 1H), 3.87 (s, 2H), 3.28 (q, $J = 5.6$ Hz, 2H), 1.55 (m, 2H), 1.36–1.31 (m, 2H); ^{13}C NMR (125 MHz, $[D_6]$DMSO): $\delta = 164.8$, 152.4, 151.6, 130.4, 129.3, 128.7, 120.3, 116.4, 39.1, 27.8, 13.4; MS (ESI): calcd for $C_{22}H_{24}Cl_2N_4O_6S_2$ $[M-H]^+$: 573, found: 573.

Compounds **82**, **84**, and **87** were prepared in the same manner as described for compound **73** with the exception that 1,6-diaminohexane was replaced by amine **78**, **76**, or **85**, respectively.

Compound 82: $R_f = 0.69$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3383, 3048, 2919, 1658, 1511, 1427, 1290, 1213, 1016, 702 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 8.81–8.71$ (m, 6H), 8.55 (dd, $J = 8.5$, 2.1 Hz, 1H), 8.28 (d, $J = 8.5$ Hz, 1H), 5.31 (s, 2H), 5.23 (s, 2H), 4.92 (t, $J = 6.7$ Hz, 2H), 4.04 (t, $J = 6.7$ Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 165.9$, 153.3, 152.9, 139.9, 131.6, 130.4, 130.2, 129.8, 129.6, 128.1, 121.4, 117.5, 39.8, 36.7, 31.5, 28.9.

Compound 84: $R_f = 0.65$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3196, 1658, 1507, 1431, 1290, 1213, 1017, 691 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 8.83–8.71$ (m, 5H), 8.67 (t, $J = 7.0$ Hz, 1H), 8.54 (dd, $J = 8.2$, 2.0 Hz, 1H), 8.28 (d, $J = 8.2$ Hz, 1H), 5.31 (s, 2H), 4.87–4.82 (m, 4H), 4.41 (t, $J = 7.3$ Hz, 2H), 3.32–3.30 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD): $\delta =$ 162.1, 153.5, 152.5, 138.1, 131.5, 130.4, 130.3, 130.1, 129.8, 127.1, 121.5, 117.5, 39.4, 31.8, 30.3, 28.9; HRMS (MALDI-FTMS): calcd for $C_{18}H_{19}ClN_2O_3S_2$ $[M+H]^+$: 379.0878, found: 379.0873.

Compound 87: $R_f = 0.63$ (silica gel, EtOAc/hexanes 1:1); IR (film): $\tilde{\nu}_{max} =$ 3393, 2931, 1658, 1510, 1429, 1289, 1219, 1014, 950, 701 cm^{-1}; ^1H NMR

(400 MHz, CD_3OD): $\delta = 7.31-7.24$ (m, 3H), 7.21–7.18 (d, $J = 5.7$ Hz, 3H), 7.09 (dd, $J = 6.6$ Hz, 1H), 6.84 (d, $J = 6.6$ Hz, 1H), 3.85 (s, 2H), 3.32 (t, $J = 6.5$ Hz, 2H), 2.66 (t, $J = 6.5$ Hz, 2H), 1.68–1.52 (m, 4H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 164.8, 152.4, 151.6, 142.6, 130.4, 129.3, 128.6, 128.4, 128.3, 125.7, 120.3, 116.4, 39.0, 35.4, 29.0, 28.8, 27.8$.

Antibacterial assay: Determination of MIC values was conduced as described in ref. [1].

In vitro toxicity assay: Baby hamster kidney BHK-21 cells (ATCC CCL10) were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal serum (FBS, Life Technologies), 2 mM glutamine, 1 × tryptose phosphate broth (TPB, Life Technologies), 1 mM sodium pyruvate (Life Technologies) and 0.5% glucose. Before the addition of test compounds exponentially growing cells were dispensed into the wells of a 96-well microtiter plate at concentration of 5000 cells per well (200 μL of DMEM without phenol-red, 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate and sodium bicarbonate). Jurkat cells were maintained in RMPI containing 10% FBS without phenol red. Prior to the addition of test compounds, exponentially growing Jurkat cells were inoculated at an initial concentration of 12000 cells per well (same medium as BHK-21 cells). 12 h after cell seeding, test compounds at varying concentrations (2 μL) were added to the 96-well plates. After 24 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the plates were analyzed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) cell proliferation assay. MTT was dissolved at a concentration of 5 mg mL^{-1} in Hank's salt solution and filtered through a 0.45 μ filter. 20 μL of this MTT solution was then added to each well including the control wells containing no cells. After incubation for 4 h, the 96-well microtiter plates were centrifuged at 2000 rpm for 10 min, the growth medium was then removed and DMSO (200 μL) added to each well. After agitation, absorbance of the wells was quantified using a scanning well microculture plate reader at test and reference wavelengths of 550 and 620 nm, respectively. The IC_{50} was defined as the concentration of the test compound that produced 50% reduction of absorbance as compared with untreated control cells.

DNA gyrase assay: The compound to be assayed (3 μL, final concentration of compound as shown in Figure 4) was added to a buffer solution (27 μL of 35 mM Tris·HCl, 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spemidine, 1 mM ATP, 6.5% (*w*/*v*) glycerol and 0.1 mg mL^{-1} bovine serum albumin) containing 0.5 μg of relaxed pBR322 (TopoGEN) and DNA gyrase (5 U, TopoGEN). This reaction mixture was incubated at 37 °C for 1 h, before being quenched by the addition of a stop solution (3 μL of 0.3% SDS, 0.3% bromophenyl blue, 40% sucrose). The DNA topoisomers were separated by electrophoresis on an 0.8% agarose gel. After development of the gel, the DNA topoisomers were visualized by staining with ethidium bromide (1 μg mL^{-1}) and examination under UV light.

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