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Bis(indole) alkaloids as sortase A inhibitors from the sponge *Spongosorites* sp.

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Abstract—A new bis(indole) alkaloid (**9**) of the hamacanthin class along with the previously reported compounds of the related structural classes, topsentin class (**1**–**4**) and hamacanthin class (**5**–**8**), was isolated from the marine sponge *Spongosorites* sp. and their inhibitory activities toward sortase A (SrtA) that play key roles in cell-wall protein anchoring and virulence in *Staphylococcus aureus* were evaluated. Our studies have identified a series of SrtA inhibitors, providing the basis for further development of potent inhibitors. The preliminary structure–activity relationship, to elucidate the essential structural requirements, has been described. The fibronectin-binding activity data highlight the potential of these compounds for the treatment of *S. aureus* infections via inhibition of SrtA activity.

Gram-positive pathogenic bacteria display surface proteins that play pivotal roles in the adhesion to specific organ tissues, invasion of host cells, or the evasion of host-immune responses.[1] These virulence-associated proteins are covalently anchored to bacterial cell wall peptidoglycan through a general sorting mechanism catalyzed by a superfamily of membrane-associated transpeptidases termed sortases.[2] In the case of *Staphylococcus aureus*, sortase A (SrtA) plays a critical role in the pathogenesis by modulating the ability of the bacterium to adhere to host tissue via the covalent anchoring of adhesions and other virulence-associated proteins to cell wall peptidoglycan.[3,4] *S. aureus* mutants lacking SrtA fail to display surface proteins and are defective in the establishment of infections without affecting microbial viability.[5] Therefore, inhibitors of SrtA might consequently be promising candidates for the treatment and/or prevention of gram-positive bacterial infections. Currently, there have only been a few reports in the literature describing inhibitors of sortase,[6–9] due, in part, to the fact that importance of sortase as a new target has only recently been acknowledged.

Indole-containing alkaloids have been frequently isolated from diverse marine invertebrates including bryozoans, coelenterates, sponges, and tunicates. Bis(indole) alkaloids, consisting of two indole moieties connected to each other via heterocyclic units, have been particularly abundant within sponges. Since topsentin (=topsentin B1), a bis(indolyl)imidazole, and its analogs were isolated from the sponge *Topsentia genitrix* (=*Spongosorites genitrix*),[10–12] metabolites containing bis(indole) moiety have been found with various carbon skeletons and functionalities.[13–20] These compounds exhibited a wide spectrum of pharmacological activities such as cytotoxic, antiviral, antimicrobial, and antiinflammatory activities as well as binding to α1 adrenergic receptor that made bis(indole) alkaloids attractive targets for biomedical and synthetic studies[21–26] (see Fig. 1).

During the course of our search for SrtA inhibitors from marine organisms, we encountered the bright yellow sponge *Spongosorites* sp. (Order Halichondrida, Family Halichondriidae) whose crude extract exhibited significant inhibitory activity (IC_{50} 45 μg/mL) toward SrtA.[27]

Keywords: Bis(indole) alkaloids; Sponge; Sortase A inhibitory activity.
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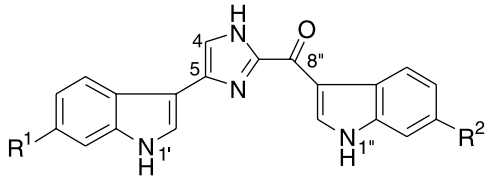
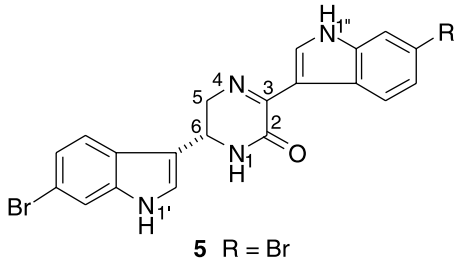
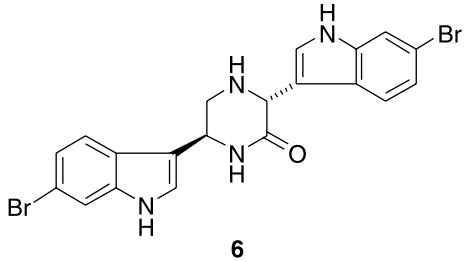


1 $R^1 = R^2 = H$
2 $R^1 = Br$, $R^2 = H$
3 $R^1 = Br$, $R^2 = OH$
4 $R^1 = Br$, $R^2 = H$, 4,5-dihydro



5 R = Br
8 R = H



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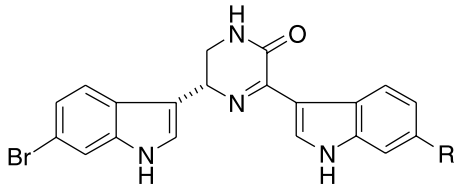

7 R = Br
9 R = H

Figure 1. Structures of bis(indole) alkaloids.

Bioassay-guided separation of the crude extract using various chromatographic techniques yielded several indole-containing compounds. Herein, we report the isolation and structure determination of a new metabolite of the hamacanthin class. In addition, biological activities of these compounds along with previously reported bis(indole) alkaloids are described.

Spongosorites sp. was collected by SCUBA at 15–20 m depth off the shore of Chuja-Do (Island), South-western Korea, in October 2003. The specimens were small pieces ($55 \times 30 \times 25$ mm) and associated with other sponges. The color was bright yellow in life and turned into dark brown in alcohol. Texture was hard and compressible. Dense ectosomal crust has many small oxeas (70–250×4–8 mm). Choanosome consists of mainly large oxea (380–1000×8–20 mm). The fresh collection was immediately frozen and kept at −25 °C until chemically investigated.

The specimens were lyophilized (dry wt 0.49 kg), macerated, and repeatedly extracted with MeOH ($2\ L \times 2$) and CH_2Cl_2 ($2\ L \times 2$). The combined crude extract (24.4 g) was partitioned between *n*-BuOH and H_2O, and then the former layer (7.0 g) was re-partitioned between 15% aqueous MeOH and *n*-hexane. The aqueous MeOH layer (5.4 g) was separated by C_{18} reversed-phase vacuum flash chromatography using sequential mixtures of MeOH and H_2O as eluents. The combined fractions (920 mg) eluted with 30 and 20% aqueous MeOH were separated by C_{18} reversed-phase HPLC (YMC ODS-A column, 35% aqueous MeOH) to yield in the order of elution, compounds **3**, **1**, **6**, **4**, **9**, and **8** as yellow amorphous solids, which were then re-purified by reversed-phase HPLC (50, 45, and 35% aqueous MeCN for **3** and **9**, **1**, and **4** and **6**, respectively). The combined fractions (890 mg) eluted with 10% aqueous MeOH and 100% MeOH from vacuum flash chromatography was separated by reversed-phase HPLC (20% aqueous MeOH) to afford in the order of elution **3**, **1**, **6**, **9**, **8**, **2**, **7**, and **5**, and then re-purified by reversed-phase HPLC (20% aqueous MeCN for **2**, **5**, and **7**). The overall purified metabolites were isolated in the following amounts: 517.4, 320.3, 37.0, 16.8, 18.1, 12.9, 22.0, 10.7, and 7.0 mg for **1**–**9**, respectively.

Based upon the results of combined spectral analyses and comparison of spectral data with those of known compounds, the major metabolites were identified to be deoxytopsentin (**1**)[11,12] and bromodeoxytopsentin (**2**).[19,20] The minor metabolites were also identified as bromotopsentin (**3**),[11,12] 4,5-dihydro-6″-deoxybromotopsentin (**4**),[12] hamacanthin A (**5**),[16] *trans*-4,5-dihydrohamacanthin A (**6**),[20] and hamacanthin B (**7**).[16] Spectral data for these compounds were in good agreement with those reported previously.

Compound **8** was isolated as an optically active amorphous solid (mp 155–157 °C; $[\alpha]_D^{25}$ −19.8° (*c* 0.3, MeOH)) which was analyzed for $C_{20}H_{15}BrN_4O$ by the HRFABMS analysis. The presence of two indole moieties was evident from the observation of 16 carbon signals in the region of δ 140–110 in the ^{13}C NMR data as well as the corresponding proton signals at δ 8.4–7.1 in the ^1H NMR data (Table 1). A combination of ^1H COSY, *g*HSQC, and *g*HMBC experiments revealed that the indoles were 3-substituted and 6-bromo-3-substituted indoles, respectively.

The remaining portion of the molecule, having the unit formula of $C_4H_4N_2O$, was determined to form the partial structure of 3,6-disubstituted-5,6-dihydro-1(2*H*)-pyrazinone by combined 2D NMR analysis. Long-range couplings between the ring protons and nearby indole carbons in the *g*HMBC data located the indole and 6-bromoindole moiety at C-3 and C-6 of the pyrazinone ring, respectively. Thus, the structure of compound **8** was determined to be the 6″-debromo derivative of hamacanthin A (**5**).[16] Based upon the comparison of the specific rotation with those of other metabolites, the

Table 1. ^{1}H and ^{13}C NMR assignments for compounds **8** and **9**

Position	**8**		**9**	
	δ_H	δ_C	δ_H	δ_C
2		160.6 C		169.1 C
3		159.5 C		160.5 C
5	4.21, dd (16.2, 4.9)	54.8 CH_2	3.76, dd (13.0, 4.7)	45.1 CH_2
	4.14, dd (16.2, 9.9)		3.59, dd (13.0, 9.5)	
6	5.05, dd (9.9, 4.9)	48.4 CH	5.30, dd (9.5, 4.7)	55.6 CH
2′	7.30, br s	125.2 CH	7.27, d (0.6)	124.7 CH
3′		114.0 C		116.1 C
3a′		126.0 C		126.5 C
4′	7.61, d (8.5)	121.3 CH	7.64, d (8.5)	121.5 CH
5′	7.17, dd (8.5, 1.6)	123.4 CH	7.15, dd (8.5, 1.5)	123.1 CH
6′		116.3 C		116.1 C
7′	7.55, d (1.6)	115.5 CH	7.56, d (1.5)	115.4 CH
7a′		139.2 C		139.4 C
2″	8.32, s	132.9 CH	8.35, s	132.9 CH
3″		112.6 C		112.7 C
3a″		127.5 C		127.5 C
4″	8.33, br d (8.0)	123.4 CH	8.44, br d (8.0)	123.8 CH
5″	7.12, ddd (8.0, 8.0, 0.9)	121.9 CH	7.09, ddd (8.1, 8.0, 0.9)	121.9 CH
6″	7.18, ddd (8.1, 8.0, 1.0)	123.6 CH	7.17, br dd (8.1, 8.1)	123.6 CH
7″	7.41, br d (8.1)	112.4 CH	7.41, br d (8.1)	112.4 CH
7a″		138.0 C		138.1 C

Measured in CD_3OD solutions. Assignments were aided by a combination of ^{1}H COSY, *g*HSQC, and *g*HMBC experiments.

stereochemistry at C-6 asymmetric center was assigned as *R*.[16,20] However, literature survey revealed that this compound was reported as a metabolite of the sponge *Spongosorites* sp. during the preparation of this manuscript.[26]

The molecular formula of compound **9**, an amorphous yellow solid (mp 210–212 °C; $[\alpha]_D^{25}$ −100.0° (*c* 0.2, MeOH)), was deduced as $C_{20}H_{15}BrN_4O$ on the basis of combined HRFABMS and ^{13}C NMR analyses. The spectral data for this compound were very similar to those obtained for compound **8**; IR (NaCl) ν_{max} 3400 (broad), 1655, 1570 cm^{-1}; UV λ_{max} (log ε) 217 (4.00), 224 (3.94), 274 (3.46), 328 (3.70) nm. In particular, signals of protons and carbons of the indole moieties in the NMR data were almost identical to each other, indicating the presence of the same 3-substituted and 6-bromo-3-substituted indoles as **8** in **9** (Table 1). The most noticeable differences in the NMR data were the chemical shifts of the protons and carbons at C-5 and C-6 of the pyrazinone ring. A combination of 2D NMR analyses revealed that the pyrazinone moiety of **9** was indeed 5,6-dihydro-4(2*H*)-pyrazinone bearing the carbonyl carbon at C-3 of the ring. The crucial evidence for this interpretation was the *g*HMBC correlations between the carbons at δ 160.5 (C-3) and 159.1 (C-2), and the protons at δ 3.76 (H-5) and 5.30 (H-6), respectively. Long-range correlations of the ring carbons with H-2′ and H-2″ of the indole moieties established the attachment of the indole and 6-bromoindole at C-2 and C-6, respectively, of the pyrazinone moiety. Thus, the structure of compound **9** was determined to be the 6″-debromo derivative of hamacanthin B (**7**).[16] Stereochemistry at the C-6 asymmetric center was assigned as *R* on the basis of comparison of the specific rotation with those of other hamacanthins.[16,20,26]

The compounds **1**–**9** were evaluated for their inhibitory activities toward *S. aureus* SrtA according to a previously documented procedure.[9,28] The inhibitory potencies, expressed as the IC_{50} values, of the tested compounds are shown in Table 2 and are compared with that of a known SrtA inhibitor, β-sitosterol-3-*O*-glucopyranoside (IC_{50}: 18.3 ± 0.37 μg/mL).[8] The results in the present study demonstrated that three bis(indole) alkaloids (**1**, **2**, and **3**) exhibited almost the same inhibitory activity upon SrtA as the β-sitosterol-3-*O*-glucopyranoside, whereas three compounds (**4**, **6**, and **9**) showed little activity (IC_{50} > 100 μg/mL).

Among the topsentins (**1**–**4**), deoxytopsentin (**1**) showed the most potent SrtA inhibitory activity (IC_{50}: 15.67 ± 0.54 μg/mL). Interestingly, the 4,5-dihydrogenation of imidazole ring (compound **4**) led to a total loss of SrtA inhibitory activity (>100 μg/mL). These results suggest that the SrtA inhibitory activities of the topsentins are greatly affected by the substitution on the

Table 2. Inhibitory effects of compounds **1**–**9** on the activity of SrtA enzyme and bacterial growth of *S. aureus* strain Newman

Compounds	SrtA IC_{50} (μg/mL)[a]	MIC (μg/mL)
1	15.67 ± 0.54	6.25
2	19.44 ± 0.02	100
3	16.70 ± 0.53	25
4	>100	>100
5	86.34 ± 1.23	3.12
6	>100	12.5
7	68.98 ± 0.69	6.25
8	34.04 ± 0.08	6.25
9	>100	100
β-Sitosterol-3-*O*-glucopyranoside	18.3 ± 0.37	200

[a] IC_{50} values are means ± SD (n = 3).

imidazole ring. Bis(indole) compounds of the hamacanthin class (**5**–**9**) also exhibited SrtA inhibitory activity. 6″-Debromohamacanthin A (**8**) showed moderate inhibitory activity (IC_{50}: 34.04 ± 0.08 μg/mL). However, insertion of a bromine atom at the C-6″ position (compound **5**) resulted in a dramatic decrease of inhibitory activity (IC_{50}: 86.34 ± 1.23 μg/mL). In addition, the 3,4-dihydrogenation of the pyrazinone ring (compound **6**) led to a total loss of activity (>100 μg/mL). Compound **8** containing a 3,6-disubstituted pyrazinone ring was significantly more active than **9** containing a 3,5-disubstituted pyrazinone ring. These results also demonstrate that, the presence of 3,6-disubstituted-5, 6-dihydro-1(2*H*)-pyrazinone skeleton and the substitution on the indole ring are important factors for the SrtA inhibitory activity of the compounds of the hamacanthin class.

An active sortase enzyme is required for the attachment of *S. aureus* to eukaryotic cell matrices. Mutant *S. aureus* strains lacking a functional sortase cannot bind to cell–matrix proteins, such as fibrinogen and fibronectin, and also cannot effect protein A-mediated binding of IgG in vitro.[29] We hypothesized that SrtA inhibitor should inhibit SrtA activity in vivo and in turn reduce fibronectin-binding protein surface display. To test this hypothesis, we employed an assay[30,31] in which cell adhesion to fibronectin-coated plates was quantified by measuring the absorbance following staining with crystal violet. It is also well known that SrtA inhibitors should act as antiinfective agents and disrupt the pathogenesis of bacterial infections without affecting microbial viability.[5] To rule out effects of test compounds on *S. aureus* (strain Newman) cell adhesion to fibronectin due to inhibition of cell growth, the minimal inhibitory concentration (MIC) was determined by the microtiter broth dilution method.[32] From the MIC test data (Table 1), compound **2** (MIC: 100 μg/mL) was selected and used in the fibronectin-binding assay. To begin with, the capacities of *S. aureus* strains Newman ($srtA^+$) as well as its isogenic knockout mutant SKM12 ($srtA^-$) to adhere to fibronectin-coated surfaces were investigated.[33] As shown in Fig. 2A, the fibronectin-binding activity of SKM12 was significantly reduced as compared with that of wild type (Newman). Treatment of strain Newman with bromodeoxytopsentin (**2**) reduced the capacity of the bacterium to adhere to fibronectin-coated surfaces in a dose-dependent manner (0–40 μg/mL) (Fig. 2B). It is important to note that the onset and magnitude of inhibition of fibronectin-binding in *S. aureus* treated with bromodeoxytopsentin (**2**) (>20 μg/mL) is comparable to the behavior of untreated SKM12.

In conclusion, bis(indole) alkaloids of the topsentin and hamacanthin classes were evaluated for their SrtA inhibitory activities. The imidazole and pyrazinone skeletons proved to be important factors for SrtA activity, changing selectivities and IC_{50}s, when compared with the structures of isolated bisindole alkaloids. The fibronectin-binding activity data highlight the potential of these compounds for the treatment of *S. aureus* infections via inhibition of sortase activity.

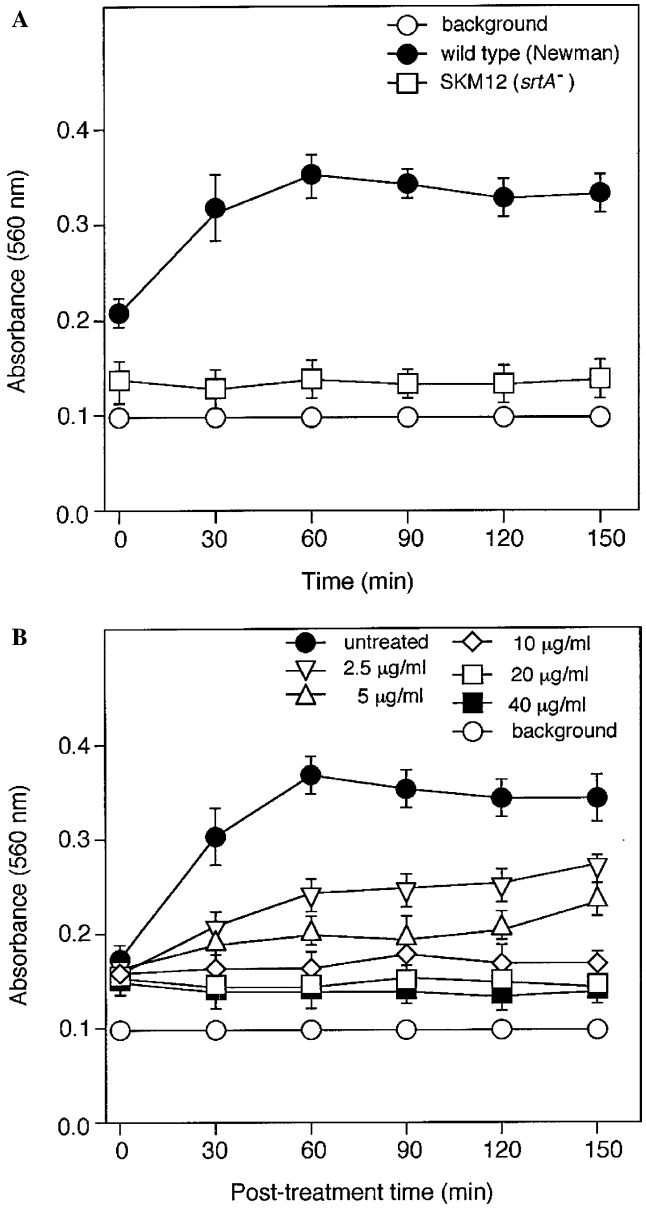

Figure 2. *Staphylococcus aureus* adhesion to fibronectin via fibronectin-binding protein. (A) Adhesion of *S. aureus* strain Newman ($srtA^+$) as well as its isogenic knockout mutant SKM12 ($srtA^-$) to fibronectin. (B) Inhibition of *S. aureus* (strain Newman) adhesion to fibronectin by bromodeoxytopsentin (**2**). The values are means ± SD ($n = 3$).

Acknowledgments

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27. Taxonomic identification: the sponge specimen was identified by C.J.S. on the basis of macro- and micro-morphological feature analysis. A voucher specimen (registry No. Spo. 45) is currently on deposit at the Natural History Museum, Hannam University, Korea, under the curatorship of C.J.S.
28. SrtA inhibition assay: reactions were performed in 300 μl containing 50 mM Tris–HCl, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.5, 55 μg recombinant $SrtA_{\Delta 24}$, 0.75 μg fluorescent peptide (Dabcyl-QALPETGEE-Edans), and prescribed concentration of test sample. Each of the test compound was dissolved in dimethylsulfoxide (DMSO), and diluted with sterilized distilled water before use (final 0.5% DMSO), which was found to have no effect on the enzyme activity when at a concentration of less than 1%. Appropriate blanks contained all of the above, with the exception of the test sample. Reactions were carried out for 1 h at 37 °C and analyzed fluorometrically by 350 nm for excitation and 495 nm for recordings.
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31. Fibronectin binding assay: *S. aureus* strain was grown in tryptic soy broth (TSB) at 37 °C to midlog phase (OD_{600} = 0.5). The culture was split into 5 ml aliquots, and test compound, or control treatment, was added as indicated. Every 30 min for 2.5 h following the addition of test compound, 0.65 ml cell suspension was removed and pelleted by centrifugation (10,000*g* for 10 min). After storing overnight at −20 °C, pellets were resuspended in 0.65 ml phosphate-buffered saline (PBS) and distributed in 100 μl aliquots to individual wells of fibronectin-coated flat-bottomed 96-well microtiter plates. Following a 2-h incubation at 37 °C, the cell suspension was removed and wells were washed with 0.15 ml PBS. Bound cells were then fixed by incubation for 30 min with 2% (v/v) glutaraldehyde. Following a second wash with PBS, cells were stained for 15 min with 0.1 ml crystal violet dye (12.5 g/L). Plates were washed again with PBS, covered with aluminum foil, and allowed to dry overnight (12–16 h). The absorbance at 560 nm was subsequently measured using a microtiter late reader.
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