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# Anti-MRSA Actinomycins $D_1$-$D_4$ from the Marine Sponge-associated *Streptomyces* sp. LHW52447

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**ABSRACT**: Actinomycins $D_1$–$D_4$ (**1**–**4**), four new D-type actinomycin analogues, were isolated from the fermentation broth of a strain of marine sponge-associated *Streptomyces* sp. LHW52447, together with actinomycin D (**5**). The structures of **1**–**4** were determined by a combination analysis of HRMS and NMR spectroscopic methods, and their absolute configurations of amino acids were determined by Marfey's analysis. Actinomycins $D_1$ (**1**) and $D_2$ (**2**) are the first two naturally occurring actinomycins with incorporation an oxazole unit into the central phenoxazinone chromophore. Both **1** and **2** showed more potent antibacterial activities against three strains of pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA) with MIC values of 0.125–0.25 μg/mL than those of **3–5** with MIC values of 0.5–1.0 μg/mL, which suggested that the anti-MRSA activity might be enhanced by the incorporation of an additional oxazole unit. In addition, the cytotoxicity evaluation against WI-38 human diploid lung fibroblasts revealed that the incorporation of oxazole unit could decrease the cytotoxicity of actinomycins on human normal cells.

**Keywords:** Actinomycins; Marine Sponge; *Streptomyces* sp.; Structural elucidation; Anti-MRSA activity.

## 1. Introduction

Actinomycins are a well-known class of chromopeptide isolated from many species of *Streptomyces*.[1] These metabolites consist of two cyclic pentapeptide lactones ($\alpha$- and $\beta$-rings) attached to a phenoxazinone chromophore through amide bonds. About 34 naturally occurring actinomycins have been reported so far,[2] while more than 40 structural analogues have been obtained from precursor-directed biosynthesis and synthetic efforts.[3] This natural product class shows very potent antitumor and antibacterial activities and remains the subject of ongoing research.[2,4,5] These chromopeptides act as intercalators with the DNA double helix.[6] The phenoxazinone chromophore fits between guanine/cytosine base pairs, while the peptidolactone side chains lying inside the minor groove of the helix.[7] With some notable exceptions, the structure elucidation of several actinomycins relies on mass spectroscopic analyses, with absolute amino acid configurations often going unassigned.[8,9] Such incomplete assignments could make it difficult to determine the structure-activity relationship between structural analogues.

In an ongoing investigation of the chemical diversity of marine sponge-associated microbes,[10] our laboratory has accumulated an extensive library of microbial isolates assembled from the marine sponges collected from the South China Sea. A strain of *Streptomyces* sp. LHW52447, isolated from the marine sponge *Phyllospongia foliascens*, was cultivated on a range of media, and metabolite production was profiled by HPLC-DAD-MS analysis. The analysis revealed an array of interesting metabolites (*m*/*z* >1200) in an organic extract derived from ISP2 broth medium.

Scaled up cultivation followed by solvent extraction and partitioning, and reversed phase chromatography, yielded a series of chromopeptides, actinomycins $D_1$–$D_4$ (**1**–**4**) and actinomycin D (**5**) (Figure 1). In structural characterization of **1**–**4** we demonstrated a workflow capable of complete structure assignments, including absolute configurations for all amino acid residues.

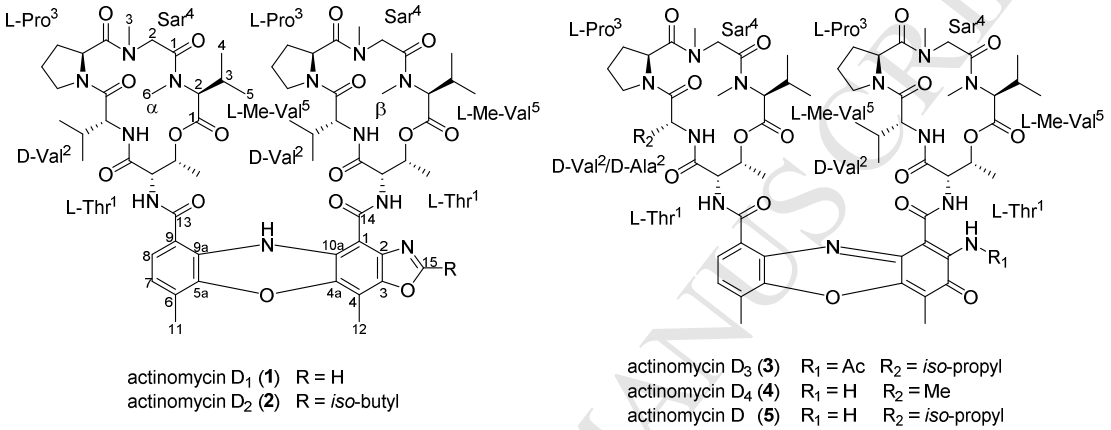


**Figure 1**. Actinomycins $D_1$–$D_4$ (**1**–**4**) and actinomycin D (**5**) from the sponge-associated *Streptomyces* sp. LHW52447.

## 2. Results and discussion

Compound **1** was isolated as orange amorphous powders. The HRESI(+)MS analysis of **1** showed a sodium adduct ion consistent with a molecular formula of $C_{63}H_{86}N_{12}O_{16}$ requiring 27 double-bond equivalents (DBE). The $^{1}H$ and $^{13}C$ NMR data for **1** (Tables 1 and 2) displayed typical features of an actinomycin compound. Detailed analysis of the $^{1}H$, $^{13}C$, COSY, HSQC, and HMBC NMR spectra allowed for the assignment of the amino acids within the $\alpha$- and $\beta$-rings as Thr, Val, Pro, Sar, and MeVal (Figure 2). The amino acid composition of both rings is identical with actinomycin D (**5**).[11] However, the 12 amu difference between **1** and actinomycin D indicated adduct of a carbon atom in **1**, which was supported by an additional

aromatic methine ($\delta_C$ 151.7/$\delta_H$ 8.19, CH-15). This methine proton showed HMBC correlations with C-2 and C-3, suggesting the phenoxazinone chromophore in actinomycin D was replaced by oxazolophenoxazine in **1**. This assignment was supported by HMBC correlations of 10-NH/C-1, C-4a, C-5a, and C-9 as well as $H_3$-12/C-3, C-4, and C-4a. Furthermore, the amino acid sequences of $\alpha$-ring and $\beta$-ring in **1** were determined by the NOESY correlations depicted in Figure 2, which was confirmed by the TOF-MS/MS fragmentation (Figure S23). The absolute configurations of amino acid residues in **1** was analyzed by 6 M HCl, followed by Marfey's analysis as described previously.[10] The presence of L-Thr, D-Val, L-Pro, Sar, and L-MeVal was unambiguously determined by comparison with authentic standards (Figure S24). Thus, the structure of **1** was determined as a new member of the actinomycin family and subsequently named actinomycin $D_1$.

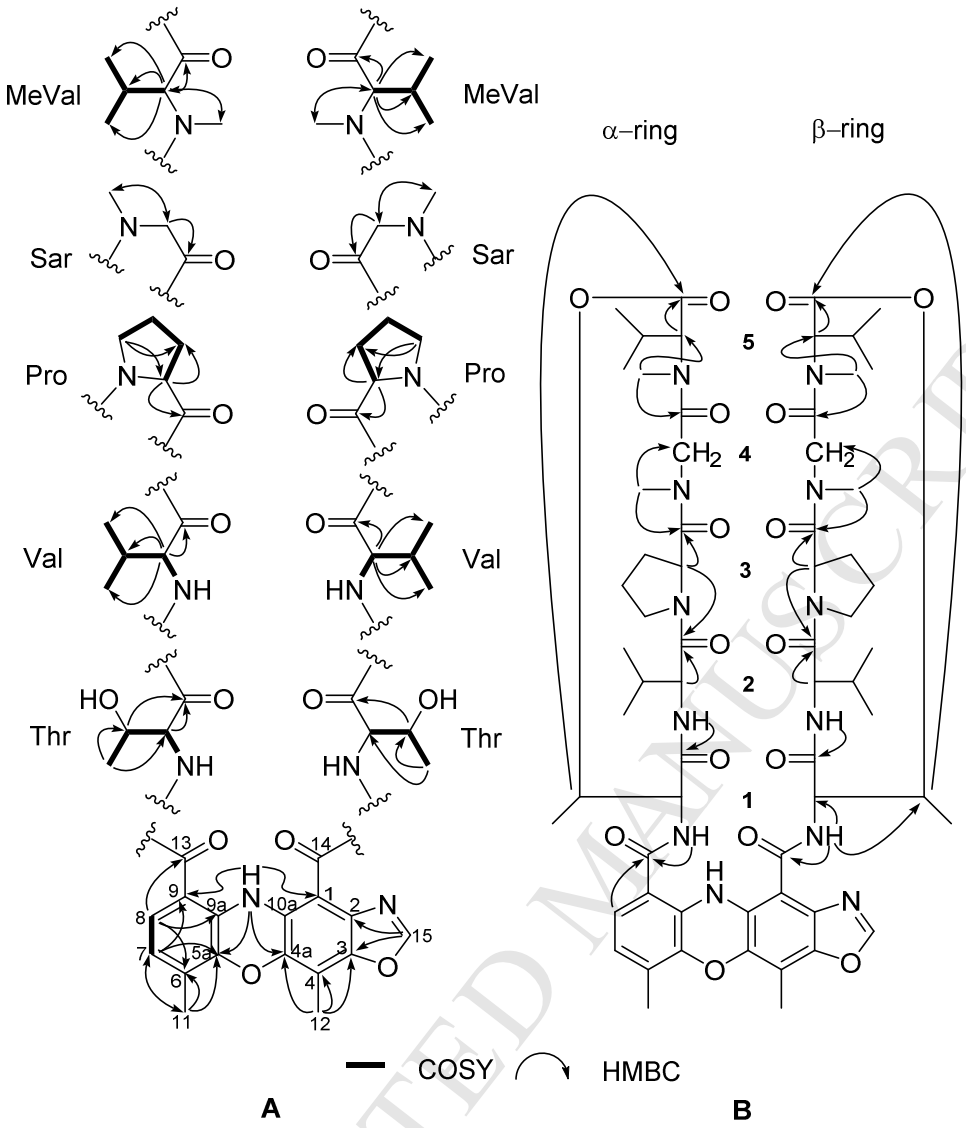


**Figure 2**. (A) Intra-residual COSY and HMBC connectivities for actinomycin $D_1$ (**1**); (B) inter-residual couplings for **1**.

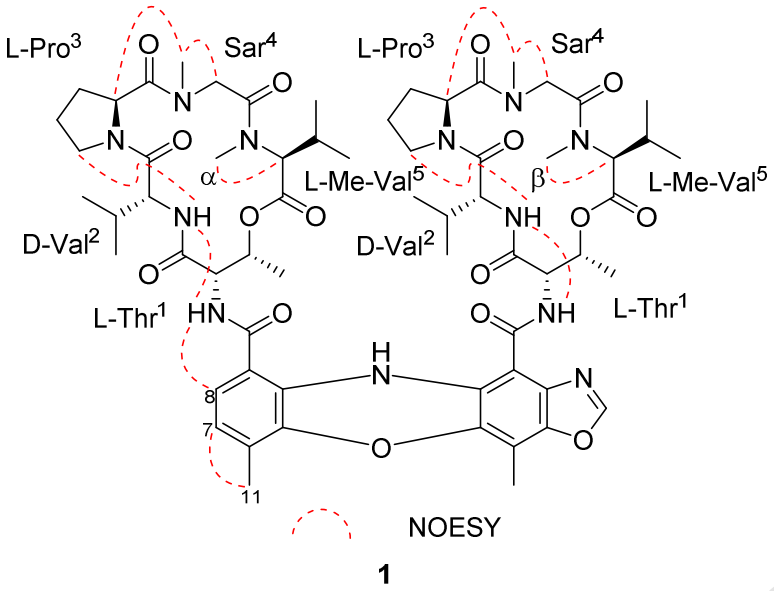


**Figure 3**. Key NOESY Correlations for **1**.

The molecular formula of **2** was determined to be $C_{67}H_{94}N_{12}O_{16}$ on the basis of HRESI(-)MS data. The NMR spectroscopic data for **2** (Tables 1 and 2) revealed characteristic features of actinomycins and were quite similar to those of actinomycin $D_1$ (**1**), except for the resonances for an *iso*-butyl group ($\delta_C/\delta_H$ 36.5/2.81, 27.1/2.23, 22.2/1.03, and 22.1/1.01) in the high field of $^1H$ and $^{13}C$ NMR spectra, supported by the COSY correlations of $H_2$-16/H-17, H-17/$H_3$-18, and H-17/$H_3$-19. Moreover, the placement of the *iso*-butyl group at C-15 ($\delta_C$ 165.8) was determined by HMBC correlations of $H_2$-16/C-15, C-18, and C-19 as well as H-17/C-15, C-16, and C-18. The sequence and absolute configurations of amino acids in **2** were established by TOF-MS/MS fragmentation (Figure S32) and Marfey's analysis (Figure S33). The overall structure of **2** was assigned as a new analogue of the actinomycin series and designated as actinomycin $D_2$.

**Table 1. $^{13}$C NMR Data (150 MHz) of 1–4**

| | pentapeptidolaction (α-ring, $\delta_C$) | | | | | pentapeptidolaction (β-ring, $\delta_C$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| no. | **1** [a] | **2** [b] | **3** [a] | **4** [a] | no. | **1** [a] | **2** [b] | **3** [a] | **4** [a] |
| L-Thr[1] | | | | | L-Thr[1] | | | | |
| 1 | 168.7, C | 169.2, C | 168.8, C | 168.6, C | 1 | 169.2, C | 169.3, C | 168.4, C | 168.9, C |
| 2 | 55.0, CH | 53.9, CH | 53.8, CH | 55.2, CH | 2 | 56.6, CH | 54.7, CH | 54.2, CH | 54.9, C |
| 3 | 70.5, CH | 72.3, CH | 73.8, CH | 75.0, CH | 3 | 71.0, CH | 72.0, CH | 73.4, CH | 75.4, CH |
| 4 | 17.1, $CH_3$ | 16.4, $CH_3$ | 16.9, $CH_3$ | 17.9, $CH_3$ | 4 | 17.5, $CH_3$ | 16.7, $CH_3$ | 16.7, $CH_3$ | 17.5, $CH_3$ |
| D-Val[2]/D-Ala[2] | | | | | D-Val[2] | | | | |
| 1 | 171.3, C | 170.6, C | 173.6, C | 173.5, C | 1 | 170.4, C | 170.6, C | 173.6, C | 173.8, C |
| 2 | 56.2, CH | 57.4, CH | 58.7, CH | 48.8, CH | 2 | 56.1, CH | 57.4, CH | 58.7, CH | 58.8, CH |
| 3 | 29.7, CH | 30.2, CH | 31.7, CH | 17.7, $CH_3$ | 3 | 30.2, CH | 30.2, CH | 31.7, CH | 32.0, CH |
| 4 | 19.2, $CH_3$ | 18.7, $CH_3$ | 18.7, $CH_3$ | | 4 | 19.3, $CH_3$ | 18.7, $CH_3$ | 18.7, $CH_3$ | 19.3, $CH_3$ |
| 5 | 18.1, $CH_3$ | 19.1, $CH_3$ | 19.3, $CH_3$ | | 5 | 18.1, $CH_3$ | 19.1, $CH_3$ | 19.2, $CH_3$ | 19.1, $CH_3$ |
| L-Pro[3] | | | | | L-Pro[3] | | | | |
| 1 | 172.9, C | 173.0, C | 173.3, C | 173.4, C | 1 | 172.6, C | 173.0, C | 173.1, C | 173.6, C |
| 2 | 55.4, CH | 54.7, CH | 55.8, CH | 56.4, CH | 2 | 55.6, CH | 54.8, CH | 55.8, CH | 56.6, CH |
| 3 | 28.5, $CH_2$ | 31.3, $CH_2$ | 31.8, $CH_2$ | 31.4, $CH_2$ | 3 | 28.1, $CH_2$ | 31.2, $CH_2$ | 31.8, $CH_2$ | 31.1, $CH_2$ |
| 4 | 24.7, $CH_2$ | 22.8, $CH_2$ | 23.3, $CH_2$ | 23.1, $CH_2$ | 4 | 24.8, $CH_2$ | 22.7, $CH_2$ | 23.3, $CH_2$ | 23.3, $CH_2$ |
| 5 | 46.8, $CH_2$ | 46.4, $CH_2$ | 47.2, $CH_2$ | 47.7, $CH_2$ | 5 | 47.0, $CH_2$ | 46.4, $CH_2$ | 47.2, $CH_2$ | 47.5, $CH_2$ |
| Sar[4] | | | | | Sar[4] | | | | |
| 1 | 170.5, C | 167.2, C | 166.5, C | 166.6, C | 1 | 169.6, C | 167.0, C | 166.3, C | 166.7, C |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 49.6, $CH_2$ | 51.2, $CH_2$ | 51.7, $CH_2$ | 51.5, $CH_2$ | 2 | 50.0, $CH_2$ | 51.1, $CH_2$ | 51.5, $CH_2$ | 51.6, $CH_2$ |
| NMe | 38.7, $CH_3$ | 34.4, $CH_3$ | 34.9, $CH_3$ | 35.1, $CH_3$ | NMe | 38.0, $CH_3$ | 34.4, $CH_3$ | 34.9, $CH_3$ | 35.3, $CH_3$ |
| L-MeVal[5] | | | | | L-MeVal[5] | | | | |
| 1 | 168.9, C | 168.2, C | 167.4, C | 167.8, C | 1 | 169.2, C | 168.1, C | 167.5, C | 167.7, C |
| 2 | 60.4, CH | 69.9, $CH_2$ | 71.6, CH | 71.2, CH | 2 | 60.5, CH | 69.5, CH | 71.5, CH | 71.4, CH |
| 3 | 27.8, CH | 26.3, CH | 26.6, CH | 27.1, CH | 3 | 28.0, CH | 26.5, CH | 26.8, CH | 27.3, CH |
| 4 | 18.8, $CH_3$ | 21.1, $CH_3$ | 21.7, $CH_3$ | 21.7, $CH_3$ | 4 | 19.1, $CH_3$ | 21.1, $CH_3$ | 21.6, $CH_3$ | 21.7, $CH_3$ |
| 5 | 18.6, $CH_3$ | 18.8, $CH_3$ | 19.1, $CH_3$ | 19.2, $CH_3$ | 5 | 18.6, $CH_3$ | 18.8, $CH_3$ | 19.1, $CH_3$ | 19.2, $CH_3$ |
| NMe | 30.5, $CH_3$ | 38.6, $CH_3$ | 39.4, $CH_3$ | 39.3, $CH_3$ | NMe | 30.5, $CH_3$ | 38.7, $CH_3$ | 39.4, $CH_3$ | 39.4, $CH_3$ |
| chromophore | | | | | | | | | |
| 1 | 101.0, C | 100.2, C | 133.1, C | 102.0, C | 10a | 133.3, C | 133.1, C | 146.9, C | 146.0, C |
| 2 | 132.4, C | 132.1, C | 132.5, C | 147.6, C | 11 | 15.2, $CH_3$ | 14.9, $CH_3$ | 14.9, $CH_3$ | 15.2, $CH_3$ |
| 3 | 143.8, C | 143.5, C | 180.7, C | 179.3, C | 12 | 9.0, $CH_3$ | 9.0, $CH_3$ | 7.8, C | 7.9, $CH_3$ |
| 4 | 112.1, C | 111.8, C | 114.4, C | 113.6, C | 13 | 168.2, C | 166.5, C | 166.7, C | 166.3, C |
| 4a | 141.3, C | 139.5, C | 145.1, C | 145.2, C | 14 | 166.8, C | 165.4, C | 162.6, C | 166.7, C |
| 5a | 141.3, C | 140.8, C | 141.8, C | 140.6, C | 15 | 151.7, CH | 165.8, C | 169.9, C | |
| 6 | 127.9, C | 127.5, C | 126.9, C | 127.8, C | 16 | | 36.5, CH | 24.1, $CH_3$ | |
| 7 | 122.3, CH | 122.1, CH | 133.6, C | 130.4, CH | 17 | | 27.1, CH | | |
| 8 | 122.3, CH | 122.1, CH | 123.4, C | 126.0, CH | 18 | | 22.2, $CH_3$ | | |
| 9 | 112.9, C | 113.6, C | 136.1, C | 132.7, C | 19 | | 22.1, $CH_3$ | | |
| 9a | 132.5, C | 131.3, C | 129.6, C | 129.3, C | | | | | |

[a]Measrued in $CDCl_3$. [b]Measured in DMSO-$d_6$.

**Table 2. $^{1}$H NMR Data (600 MHz) of 1–4**

| | pentapeptidolaction (α-ring, $\delta_C$) | | | | | pentapeptidolaction (β-ring, $\delta_C$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| no. | **1** [a] | **2** [b] | **3** [a] | **4** [a] | no. | **1** [a] | **2** [b] | **3** [a] | **4** [a] |
| L-Thr[1] | | | | | L-Thr[1] | | | | |
| 2 | 5.18, d (9.6) | 4.96, dd (9.2, 2.4) | 4.87, dd (8.9, 1.6) | 4.47, dd (6.7, 2.6) | 2 | 4.91, m | 4.89, dd (8.7, 2.3) | 4.76, d (9.7) | 4.58, dd (6.2, 2.6) |
| 3 | 5.80, qd (6.6, 2.3) | 5.14, qd (6.1, 1.9) | 5.43, qd (6.2, 1.5) | 5.17, m | 3 | 5.66, q (6.4) | 5.23, qd (6.2, 1.7) | 5.36, q (6.3) | 5.17, m |
| 4 | 1.19, d (6.4) | 1.16, d (6.4) | 1.36, d (6.2) | 1.24, m | 4 | 1.29, d (6.4) | 1.18, d (6.3) | 1.26, d (6.3) | 1.24, m |
| NH | 8.97, d (9.4) | 7.27, d (9.0) | 6.72, d (8.9) | 7.27, m | NH | 10.01, d (8.5) | 9.72, d (8.9) | 7.38, m | 7.74, d (6.8) |
| D-Val[2]/ D-Ala[2] | | | | | D-Val[2] | | | | |
| 2 | 4.36, m | 3.46, m | 3.64, m | 3.93, m | 2 | 4.49, m | 3.47, m | 3.66, m | 3.56, m |
| 3 | 1.91, m | 1.87, m | 2.14, m | 1.35, d (6.7) | 3 | 1.90, m | 1.88, m | 2.16, m | 2.19, m |
| 4 | 0.91, m | 0.92, m | 1.14, d (6.2) | 8.09, d (5.9) | 4 | 0.91, m | 0.93, m | 1.14, d (6.2) | 0.92, d (6.8) |
| 5 | 0.81, m | 0.69, [o] d (6.7) | 0.89, d (6.4) | | 5 | 0.84, m | 0.69, d (6.7) | 0.89, d (6.4) | 1.13, d (6.7) |
| NH | 6.43, d (8.4) | 8.40, d (5.8) | 8.67, d (6.0) | | NH | 7.13, d (8.5) | 8.33, d (5.8) | 8.58, d (6.2) | 8.21, d (6.3) |
| L-Pro[3] | | | | | L-Pro[3] | | | | |
| 2 | 4.89, m | 6.26, d (8.4) | 6.33, dd (9.6, 2.0) | 5.97, d (9.2) | 2 | 4.87, m | 6.20, d (7.9) | 6.24, dd (8.8, 2.4) | 5.76, d (9.0) |
| 3a | 2.05, m | 2.08, m | 2.38, m | 2.93, m | 3 | 2.05, m | 2.10, m | 2.32, m | 2.59, m |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3b | 1.92, m | 1.74, m | 1.86, m | 1.86, m | | 1.92, m | 1.74, m | 1.86, m | 1.89, m |
| 4a | 2.30, m | 1.91, m | 2.15, m | 2.27, m | 4 | 2.36, m | 1.90, m | 2.15, m | 2.20, m |
| 4b | 1.94, m | 1.67, m | 1.98, m | 2.09, m | | 1.94, m | 1.67, m | 1.93, m | 2.09, m |
| 5a | 3.91, m | 3.47, m | 3.88, m | 3.83, m | 5 | 4.11, m | 3.49, m | 3.84, m | 3.91, m |
| 5b | 3.56, m | 3.26, m | 3.64, m | 3.73, m | | 3.52, m | 3.25, m | 3.62, m | 3.73, m |
| Sar[4] | | | | | Sar[4] | | | | |
| 2a | 4.77, m | 4.77, m | 4.93, d (17.3) | 4.78, d (16.8) | 2 | 4.91, m | 4.74, m | 4.81, d (17.4) | 4.76, d (17.0) |
| 2b | 3.05, m | 4.12, d (18.0) | 3.67, m | 3.61, d (17.5) | | 3.07, m | 4.08, d (18.0) | 3.66, m | 3.68, d (17.6) |
| NMe | 3.42, s | 2.75, s | 2.90, s | 2.88, s | NMe | 3.31, s | 2.75, s | 2.90, s | 2.88, s |
| L-MeVal[5] | | | | | L-MeVal[5] | | | | |
| 2 | 4.74, m | 3.13, d (9.0) | 2.82, d (9.9) | 2.68, d (8.8) | 2 | 5.03, d (11.0) | 3.22, m | 2.80, d (9.8) | 2.69, d (9.0) |
| 3 | 2.17, m | 2.51, m | 2.74, m | 2.60, m | 3 | 2.21, m | 2.56, m | 2.74, m | 2.66, m |
| 4 | 0.84, m | 0.98, d (6.6) | 1.01, d (5.9) | 0.96, d (6.6) | 4 | 0.92, m | 0.97, d (6.6) | 1.01, d (5.9) | 0.96, d (6.6) |
| 5 | 0.78, m | 0.79, d (7.2) | 0.81, d (6.8) | 0.75, d (6.6) | 5 | 0.82, m | 0.81, d (7.2) | 0.79, d (6.7) | 074, d (6.6) |
| NMe | 3.21, s | 3.19, s | 3.18, s | 2.93, s | NMe | 3.09, s | 3.22, s | 3.02, s | 2.90, s |
| chromophore | | | | | | | | | |
| 7 | 6.58, d (7.9) | 6.64, d (8.3) | 7.39, d (8.4) | 7.36, d (7.9) | 16 | | 2.81, m | 2.16, s | |
| 8 | 7.60, d (8.2) | 7.13, d (8.1) | 7.27, d (8.4) | 7.65, d (7.5) | 17 | | 2.23, m | | |
| 11 | 2.15, s | 2.16, s | 2.48, s | 2.55, s | 18 | | 1.03, d (6.7) | | |
| 12 | 2.32, s | 2.30, s | 2.15, s | 2.24, s | 19 | | 1.01, d (6.6) | | |
| 15 | 8.19, s | | | | NH | 11.97, s | 11.77, s | | |

[a]Measrued in $CDCl_3$. [b]Measured in DMSO-$d_6$.

Compound **3** was assigned the molecular formula $C_{64}H_{88}N_{12}O_{17}$ by the HRESI(-)MS data. The NMR data showed the presence of the phenoxazinone chromophore and two identical pentapeptidolactones that contained Thr, Val, Pro, Sar, and MeVal moieties (Tables 1 and 2). Compound **3** differed with actinomycin D by an additional acetylation of 2-NH ($\delta_C$ 169.9, C-15, and $\delta_C$ 24.1/$\delta_H$ 2.16, $CH_3$-16), which was supported by the 2D NMR analysis. In combination with the TOF-MS/MS fragmentation (Figure S41) and Marfey's analysis (Figure S42), the data were consistent with structure of **3** as depicted and named actinomycin $D_3$.

The molecular formula of compound **4** was determined as $C_{60}H_{82}N_{12}O_{16}$ by the HRESI(-)MS data with less of 28 amu ($C_2H_4$) than actinomycin D. The NMR data recorded for **4** was nearly identical with those of actinomycin D, except for the replacement of Val by Ala in $\alpha$-ring of **2** (Tables 1 and 2). Further COSY and HMBC correlations were full agreement with Ala in $\alpha$-ring. Marfey's anlysis revealed the D-configuration of Ala (Figure S51). Thus the whole structure of **4** was assigned as shown and named actinomycin $D_4$.

The antibacterial activity of the isolated actinomycins was evaluated against three strains of pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA),[12] using chloramphenicol and daptomycin as positive control (Table 3), the anti-MRSA activities of **1** and **2** are nearly 2–4 times potent than those of **3**–**5**, which suggested the anti-MRSA activity might be enhanced by incorporation of an additional oxazole unit into the phenoxazinone chromophore. In addition, the cytotoxic activity of the five isolates was evaluated against WI-38 human diploid lung fibroblasts (Table 3). The results showed that **1** and **2** showed less cytotoxicity against human normal WI38 cells than **3**–**5**, which suggested that the incorporation of oxazole unit into the chromophore could more dramatically decrease the cytotoxicity of actinomycins

against human normal cells.

**Table 3.** Anti-MRSA activity and cytotoxicity of **1–5** against three strains of pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA) and WI-38 human diploid lung fibroblasts.

| Compound | MIC (μg/mL) | | | $IC_{50}$ (μM) |
|---|---|---|---|---|
| | P172 | P175 | ATCC 33591 | WI38 |
| **1** | 0.125 | 0.25 | 0.125 | 74 ± 10 |
| **2** | 0.25 | 0.25 | 0.25 | 126 ± 6 |
| **3** | 0.5 | 1.0 | 0.5 | 18 ± 2 |
| **4** | 0.5 | 0.25 | 0.25 | 23 ± 4 |
| **5** | 0.5 | 0.25 | 0.25 | 11 ± 3 |
| chloramphenicol[a] | 0.5 | 0.5 | 1.0 | |
| daptomycin[a] | 0.063 | 0.031 | 0.25 | |
| Adriamycin[a] | | | | 62 ± 22 |

[a]positive control

## 3. Conclusion

In summary, four new D-type actinomycin analogues, actinomycins $D_1$–$D_4$ (**1**–**4**), were isolated from the fermentation broth of a strain of marine sponge-associated *Streptomyces* sp. LHW52447. Antibacterial and cytotoxicity assays showed that the incorporation of oxazole unit into the phenoxazinone chromophore would enhance the antibacterial activity of actinomycins while decrease their cytotoxicity against human normal cells. This represents the first actinomycin compounds from a marine source and highlights the importance of continued efforts toward screening for chemical diversity within sponge-associated microbes.

## 4. Experimental Section

### *4.1 General Experimental Procedures*

Specific optical rotations ($[\alpha]_D$) were measured on an Autopol I polarimeter (No. 30575, Rudolph Research Analytical) with a 10 cm length cell at room temperature. NMR spectra were recorded at room temperature on a Bruker Avance DRX-600 MHz NMR spectrometer with DMSO-$d_6$ and $CDCl_3$ as the solvent and internal standard.

Spectra were referenced to residual solvent signals with resonances at $\delta_H$ 2.49/$\delta_C$ 39.5 for DMSO-$d_6$ and $\delta_H$ 7.26/$\delta_C$ 77.0 for $CDCl_3$. Column chromatography was conducted using silica gel (65 × 250 or 230 × 400 mesh). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates. Purification of the compounds was performed using a Waters Alliance 2695 separation module equipped with a Waters 2998 photodiode array (PDA) detector.

*4.2 Collection and Isolation of Streptomyces sp. LHW52447*

The *Streptomyces* sp. LHW52447 was isolated in 2013 from the specimen of marine sponge *Phyllospongia foliascens*, collected from the Xisha Islands in the South China Sea. The marine sponge sample was cut into pieces and dissected under aseptic conditions and placed on ISP2 agar plates (comprising 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$, 0.02% chloramphenicol, and 1.5% agar in 50 % sterile water). Agar plates were sealed and incubated at 26.5 °C for 3-4 weeks. A pure culture of bacterial LHW52447 was obtained by single-colony serial transfer on agar plates. Taxonomic analysis identified LHW52447 as *Streptomyces* sp.

*4.3 Fermentation and Fractionation*

The strain of *Streptomyces* sp. LHW52447 was cultivated in ten 1 L Erlenmeyer flasks, each containing 250 mL ISP2 medium. After 3 days of incubation at 28 °C with 200 rpm agitation, the cultures were used to incolate ten flasks (1 L), each containing 250 mL of ISP2 medium. The fermentation was continued for 7 days at 28 °C with 200 rpm agitation. All culture flasks were combined and extracted with EtOAc until the organic phase was colorless. The combined organic phase was concentrated *in vacuo* to yield a crude extract (9.6 g), which was subjected to VLC on silica gel using gradient of *n*-hexane/acetone (from 100:1 to 0:1) to obtain eight

fractions (A–H). HPLC-DAD-MS analysis revealed that actinomycin compounds were mainly distributed in fractions F and G. Both of the two fractions were further separated by reversed-phase MPLC (gradient MeOH/$H_2O$, from 10% to 100% for 180 min, flow rate 20 mL/min, UV detection at 210 nm), and the subfractions were further purified by semi-preparative reversed-phase HPLC (YMC $C_{18}$ RS column, 250 × 10 mm, 5 μm, 2 mL/min) to afford actinomycin $D_3$ (**3**) (45% MeCN/$H_2O$, $t_R$ = 24.1 min, 7.7 mg), actinomycin $D_4$ (**4**) (50% MeCN/$H_2O$, $t_R$ = 60.0 min, 8.3 mg), actinomycin D (**5**) (55% MeCN/$H_2O$, $t_R$ = 20.2 min, 47.5 mg), actinomycin $D_1$ (**1**) (55% MeCN/$H_2O$, $t_R$ = 27.3 min, 8.9 mg), and actinomycin $D_2$ (**2**) (60% MeCN/$H_2O$, $t_R$ = 60.1 min, 3.8 mg).

*4.3.1 Actinomycin $D_1$ (**1**)*

Orange solids; $[\alpha]_D$ -116.9 (*c* 0.1, MeOH); UV (MeOH) (log $\varepsilon$) $\lambda_{max}$ 225 (4.10), 252 (4.06), 409 (3.71) nm; IR (KBr) $\nu_{max}$ 3418, 3269, 2964, 2933 2875, 1744, 1651, 1504, 1454, 1259, 1192, 734 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, Tables 1 and 2; HRESI(+)MS *m/z* 1289.6185 $[M + Na]^+$ (calcd for $C_{63}H_{86}N_{12}O_{16}Na$, 1289.6182).

*4.3.2 Actinomycin $D_2$ (**2**)*

Orange solids; $[\alpha]_D$ -85.5 (*c* 0.15, MeOH); UV (MeOH) (log $\varepsilon$) $\lambda_{max}$ 228 (4.13), 252 (4.12), 410 (3.79) nm; IR (KBr) $\nu_{max}$ 3445, 3268, 2966, 2933, 2876, 1747, 1652, 1504, 1449, 1256, 1192, 1093 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, Tables 1 and 2; HRESI(-)MS *m/z* 1321.6829 $[M - H]^-$ (calcd for $C_{67}H_{93}N_{12}O_{16}$, 1321.6833).

4.3.2 *Actinomycin $D_3$ (**3**)*

Orange solids; $[\alpha]_D$ -51.4 (*c* 0.15, MeOH); UV (MeOH) (log $\varepsilon$) $\lambda_{max}$ 214 (3.97), 376 (3.33), 460 (3.05) nm; IR (KBr) $\nu_{max}$ 3400, 3254, 2962, 2997 2855, 1747, 1669, 1622, 1513 1470, 1299, 1192, 822, 737 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, Tables 1 and 2; HRESI(-)MS *m/z* 1295.6305 $[M - H]^-$ (calcd for $C_{64}H_{87}N_{12}O_{17}$, 1295.6312).

4.3.3 *Actinomycin $D_4$ (**4**)*

Orange solids; $[\alpha]_D$ -89.4 (*c* 0.15, MeOH)； UV (MeOH) (log $\varepsilon$) $\lambda_{max}$ 214 (3.97), 376 (3.33), 460 (3.05) nm; IR (KBr) $\nu_{max}$ 3445, 3268, 2966, 2933, 2876, 1747, 1652, 1504, 1449, 1256, 1192, 1093 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data, Tables 1 and 2; HRESI(-)MS *m/z* 1225.5887 $[M - H]^-$ (calcd for $C_{60}H_{81}N_{12}O_{16}$, 1225.5894).

*4.4 Acid Hydrolysis of **1**–**4** and Absolute Configurations of Amino Acids by Marfey's analysis*

Analysis was carried out following the published method.[10,13] Samples of compounds **1**–**4** (0.1 mg) in 6 M HCl (100 μL) were heated to 110 °C in a sealed vial for 12 h, and then the hydrolysates were concentrated under a stream of dry $N_2$. The hydrolysates were then treated with 1 M $NaHCO_3$ (40 μL) and L-FDLA (1-fluoro-2-4-dinitrophenyl-5-L-leucine amide) (1% solution in acetone, 50 μL) for 2 h, after which the reaction was neutralized with 1M HCl. Analytes and the authentic amino acid standards prepared from treatment of L-Thr, D-Val, L-Pro, L-MeVal, and D-Ala with both D-FDAA and L-FDAA, were subjected to HPLC-ESI -MS analysis. The retention times of the L- and L-/D-FDLA-derivatized amino acids were measured by UPLC-MS using an Acquity UPLC BEH $C_{18}$ column (2.1 × 50 mm, 1.7 μm, 0.5 mL/min) with a linear gradient from 10% to 100% MeCN/$H_2O$ containing 0.1% formic acid over 10 min. The absolute configurations of amino acids were established by comparison of the retention times of the L- and D-FDLA derivatives of corresponding amino acids. The retention times of the L-FDLA amino acid derivatives were 3.07 min (L-Thr, *m/z* 414 $[M + H]^+$), 4.82 min (D-Val, *m/z* 412 $[M + H]^+$), 3.59 min (L-Pro, *m/z* 410 $[M + H]^+$), 4.36 min (L-MeVal, *m/z* 426 $[M + H]^+$), and 4.06 min (D-Ala, *m/z* 384 $[M +H]^+$).

*4.5 Cytotoxicity Assay*

Cytotoxicity of the five isolates were evaluated against human lung WI38 embryonal firoblasts using a reported protocol after slight modifications.[14] Cells were cultured in 96-well plates in 80 μL of medium in a humidified 37 °C incubator supplied with 5% $CO_2$. The test compounds were added in a 25 μL aliquot generated by serial dilution in serum-free medium on the day of the experiment, after prior removal of 25 μL of media from the treated well. Aliquots were generated from stock solutions of 6 mg/mL compound in 100% DMSO. The test compounds were evaluated in WI38 cells for 48 h treatment. Each compound was tested at final concentrations ranging from 0.1 to 30 μM. In all cases, cell viability was determined after 48 h treatment using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cytotoxicity of each test compound was assessed in at least three independent cultures. The $IC_{50}$ values were derived by nonlinear regression analysis.

*4.6 Antibacterial Assay*

Minimum inhibitory concentration (MIC) was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[11] The $MIC_{50}$ values were recorded using a spectrophotometer. For antibiotic sensitivity assays, bacteria in 96-well plates (Corning) were incubated with **1**−**5** or antibiotic standards at final concentrations of 0 to 64 μg/mL. The plates were incubated at 37 °C and read at 24 h.

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Notes

The authors declare no competing financial interest.

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## Appendix A. Supplementary data

Copies of 1D and 2D NMR, HRESIMS, QTOF-MS/MS, and Marfey's analysis of **1–4**. These materials are available free of charge via the Internet at http:…

## References

(1) H. Lackner, I. Bahner, N. Shigematsu, L. K. Pannell, A. B. Mauger, J. Nat. Prod. 63 (2000), 352−356.

(2) W. Cai, X. Wang, S. I. Elshahawi, L. V. Ponomareva, X. Liu, M. R. McErlean, Z. Cui, A. L. Arlinghaus, J. S. Thorson, S. G. Van Lanen, J. Nat. Prod. 79 (2016), 2731−2739.

(3) J. Bitzer, V. Gesheva, A.Zeeck, J. Nat. Prod. 69 (2006), 1153−1157.

(4) H. Lackner, H. Hülsmann, S. Heinze, H. Simon, H. Bär, C. Zimmer, U. Gräfe, J.

Antibiot. 53 (2000), 84−87.

(5) J. Bitzer, M. Streibel, H. J. Langer, S. Grond, Org. Biomol. Chem. 7 (2009), 444−450.

(6) M. J. Waring, Sequence-Specific DNA Binding Agents; Royal Society of Chemistry, 2006; Vol. 6.

(7) S. Kamitori, F. Takusagawa, J. Am. Chem. Soc. 116 (1994), 4154−4165.

(8) U. F. Castillo, G. A. Strobel, K. Mullenberg, M. M. Condron, D. B. Teplow, V. Folgiano, M. Gallo, R. Ferracane, L. Mannina, S. Viel, M. Codde, R. Robison, H. Porter, J. Jensen, FEMS Microbiol. Lett. 255 (2006), 296–300.

(9) U. F. Castillo, G. A. Strobel, E. J. Ford, W. M. Hess, H. Porter, J. B. Jensen, H. Albert, R. Robison, M. A. Condron, D. B. Teplow, D. Stevens, D. Yaver, Microbiology 148 (2002), 2675–2685.

(10) L. J. Ding, W. Yuan, X. J. Liao, B. N. Han, S. P. Wang, Z. Y. Li, S. H. Xu, W. Zhang, H. W. Lin, J. Nat. Prod. 79 (2016), 2045–2052.

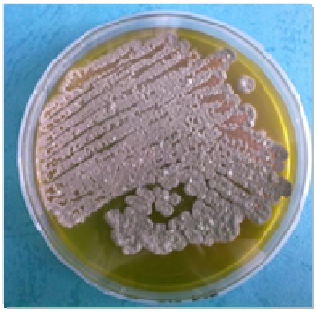
(11) Q. Wang, Y. Zhang, M. Wang, Y. Tan, X. Hu, H. He, C. Xiao, X. You, Y. Wang, M. Gan, Sci. Rep. 7 (2017), 3591.

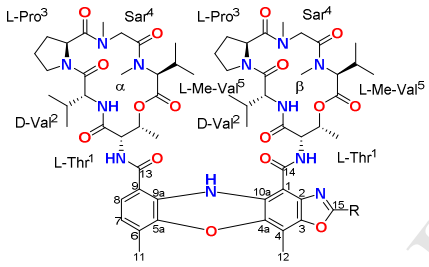
(12) D. Wang, C. Wang, P. Gui, H. Liu, S. M. H. Khalaf, E. A. Elsayed, M. A. M. Wadaan, W. N. Hozzein, W. Zhu, Front. Microbiol. 8 (2017), 1147.

(13) K. X. Zhan, W. H. Jiao, F. Yang, J. Li, S. P. Wang, Y. S. Li, B. N. Han, H. W. Lin, J. Nat. Prod. 77 (2014), 2678–2684.

(14) C. C. Thornburg, M. Thimmaiah, L. A. Shaala, A. M. Hau, J. M. Malmo, J. E. Ishmael, D. T. A. Youssef, K. L. McPhail, J. Nat. Prod. 74 (2011), 1677−1685.

# Graphical abstract






actinomycin $D_1$ (**1**) R = H
actinomycin $D_2$ (**2**) R = *iso*-butyl