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Sintokamides A to E, Chlorinated Peptides from the Sponge *Dysidea* sp. that Inhibit Transactivation of the N-Terminus of the Androgen Receptor in Prostate Cancer Cells

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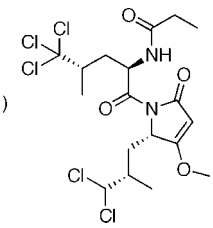
ABSTRACT

Sintokamide A (**1**)



The new chlorinated peptides sintokamides A to E (1−5) have been isolated from specimens of the marine sponge *Dysidea* sp. collected in Indonesia. Their structures were elucidated by a combination of spectroscopic and single-crystal X-ray diffraction analyses. Sintokamide A (1) is an inhibitor of N-terminus transactivation of the androgen receptor in prostate cancer cells.

The American Cancer Society has estimated that prostate cancer will kill ≈ 30 000 men in the USA in 2008. Localized prostate cancer can be cured by surgery or radiation therapy. However, the only effective treatment available for advanced disease is surgical or chemical castration to withdraw androgens, which are essential for the survival of prostate epithelial cells. Androgen ablation therapy causes a temporary reduction in tumor burden concomitant with a decrease in serum prostate-specific antigen (PSA). Unfortunately, prostate cancer will eventually begin to grow again in the absence of androgens to form castration recurrent (also called androgen independent or hormone refractory) disease. Castration recurrent disease is biochemically characterized before the onset of symptoms by a rising titer of serum PSA. Once the disease becomes castration recurrent, most patients succumb to their cancer within two years.

It has been proposed that castration recurrent prostate cancer results from activation of the androgen receptor (AR)

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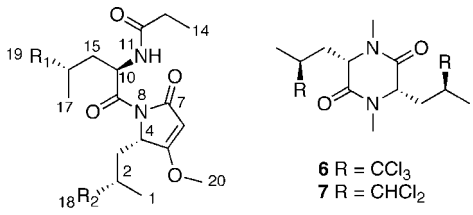
by events other than the normal binding of androgens at the ligand-binding domain. We have shown that the N-terminus domain (NTD) of the AR plays a critical role in transcriptional activation of the AR in the absence of androgens, resulting in androgen-independent proliferation of prostate cancer cells.[1] This work identified the AR NTD as a novel therapeutic target for treating castration recurrent prostate cancer with small-molecule drugs.



1 $R_1 = CCl_3$, $R_2 = CHCl_2$
2 $R_1 = CCl_3$, $R_2 = CCl_3$
3 $R_1 = CHCl_2$, $R_2 = CHCl_2$
4 $R_1 = CCl_3$, $R_2 = CH_2Cl$
5 $R_1 = CCl_3$, $R_2 = CH_3$

6 R = CCl_3
7 R = $CHCl_2$

A new assay has been employed to screen a library of marine natural product extracts for molecules that inhibit transactivation of the AR NTD in LNCaP human prostate cancer cells stably expressing the ARR3-luciferase reporter. The assay consisted of activating the endogenous AR using a synthetic androgen, R1881, and measuring levels of luciferase activity. Marine sponge extracts were added 1 h prior to the addition of R1881 to the cells and incubated for an additional 48 h before harvesting and measuring luciferase activity in the cell lysates. MeOH extracts of a *Dysidea* sp. of sponge collected in Indonesia showed promising activity in the initial screen. Assay-guided fractionation of the *Dysidea* sp. extract identified the sintokamides A to E (**1**−**5**) as the bioactive components. Details of the isolation, structure elucidation, and biological activities of the sintokamides are presented below.

Specimens of *Dysidea* sp. (140 g wet weight) were collected by hand using SCUBA at a depth of −15 m near Palau Sintok, Karimunjawa archipelago, Indonesia. The sponge specimens were cut into small pieces and extracted exhaustively with MeOH. The combined MeOH extracts were concentrated in vacuo, and the resultant oil was partitioned between EtOAc (4 × 5 mL) and H_2O (20 mL). The bioactive EtOAc-soluble materials were chromatographed on Sephadex LH-20 eluting with 4:1 MeOH/CH_2Cl_2 to give a fraction that exhibited activity in the assay. This bioactive material was further fractionated by sequential application of Si-gel flash chromatography (eluent: step gradient from 19:1 hexanes/EtOAc to EtOAc) and C_{18} reversed-phase HPLC eluted with several mixtures of MeCN/H_2O and MeOH/H_2O to give pure samples of the new peptides sintokamides A to E (**1**−**5**) and the known diketopiperazines dysamides A (**6**) and D (**7**) (Supporting Information). The structures of dysamides A (**6**) and D (**7**) were confirmed by comparing their spectroscopic data with the literature values.[2]

Sintokamide A (**1**) was obtained as an optically active clear oil ($[\alpha]^{25}_D$ +35.9) that gave an $[M + Na]^+$ ion at *m*/*z* 531.0145 in the HRESIMS (calcd for $C_{18}H_{25}N_2O_4Cl_5Na$, 531.0154) consistent with a molecular formula of $C_{18}H_{25}N_2O_4Cl_5$, requiring five sites of unsaturation. The ^{13}C NMR spectrum of **1** showed 18 well-resolved resonances in agreement with the HRESIMS measurement (Supporting Information). HSQC data obtained for **1** identified 24 hydrogen atoms attached to carbon (4 × CH_3; 3 × CH_2, 6 × CH; 5 × C), and one resonance in the ^1H NMR spectrum (δ 6.06, bs), that showed no HSQC correlation, was assigned to an exchangable hydrogen (NH-11), accounting for the 25 hydrogen atoms in the molecular formula. Correlations in the ^{15}N-lrHMQC spectrum[3] identified ^{15}N resonances at −262.6 (*N*-11) and −212.0 (*N*-8), confirming the presence of two nitrogen atoms in **1**. Four deshielded ^{13}C NMR resonances at δ 169.1 (C-7), 172.4 (C-9), 173.0 (C-12), and 179.0 (C-5) could be assigned to one oxygenated alkene and three ester/amide carbonyl carbons, and a deshielded resonance at δ 93.7 (C-6) was assigned to a second alkene carbon, accounting for four of the five required sites of unsaturation and revealing that sintokamide A (**1**) contained a ring.

COSY data obtained for **1** identified a series of isolated proton spin systems (Figure 1). The first system, which consisted of a methyl triplet at δ 1.08 (J = 7.6 Hz; Me-14) correlated to a methylene resonance at δ 1.95 (m; H-13/H13′), was assigned to an ethyl fragment. The second spin system contained a methyl resonance at δ 1.42 (d, J = 6.7 Hz; Me-17) that was correlated to a methine resonance at δ

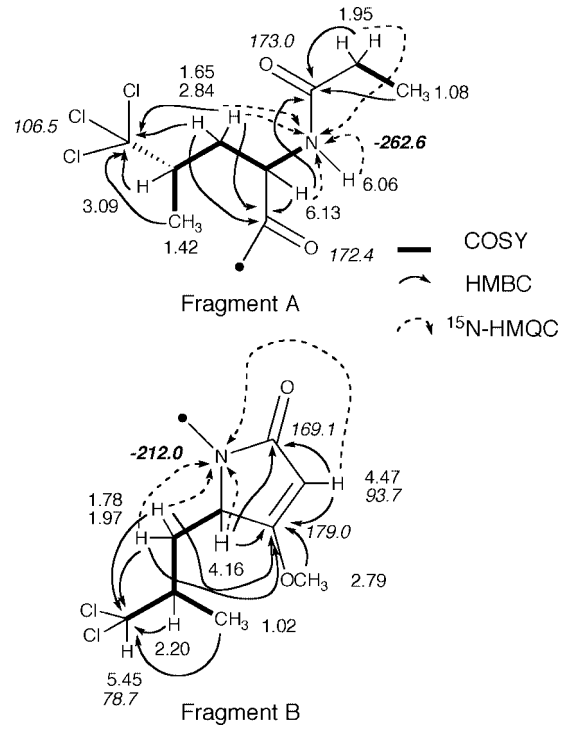


Figure 1. COSY, HMBC, and lr^{15}N-HMQC correlations observed for sintokamide A (**1**).

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3.09 (m; H-16) and from there in sequence to a pair of methylene proton resonances at δ 1.65 and 2.84 (H-15/H-15′), on to a methine resonance at δ 6.13 (m; H-10), and ending at an exchangeable proton resonance at δ 6.06 (bs; NH-11). A third system started with a methyl resonance at δ 1.02 (d, $J = 6.7$ Hz; Me-1) that was correlated to a methine resonance at δ 2.20 (m; H-2), and this methine resonance was in turn correlated to a pair of methylene proton resonances at δ 1.78 and 1.97 (H-3/H-3′) and to a methine resonance at δ 5.45 (d, $J = 2.8$ Hz; H-18). Both methylene resonances (δ 1.78/ 1.97) were further correlated to a methine at δ 4.16 (t, $J = 5.3$ Hz; H-4), which was the other terminus of the spin system. The final resonances in the ^{1}H NMR spectrum were a methyl singlet at δ 2.79 (Me-20) and an olefinic methine at δ 4.47 (s, H-6).

HMBC and ^{15}N-HMQC correlations shown in Figure 1 provided the additional information required to connect the proton spin systems to the nonprotonated functional groups in **1**. ^{15}N-HMQC correlations between the ^{1}H resonances at δ 6.06 (*N*H-11), 1.65 (H-15), 2.84 (H-15′), and 6.13 (H-10) and the ^{15}N resonance at δ −262.6 (*N*-11) demonstrated that the exchangeable proton (δ 6.06) at the terminus of this ^{1}H spin system was attached to a nitrogen atom. HMBC correlations between both the methylene proton resonance at δ 1.95 (H_2-13) and the methine resonance at δ 6.13 and the carbonyl resonance at δ 173.0 showed that the nitrogen atom also contained a propionyl substituent. A second series of HMBC correlations between the proton resonances at δ 1.65 (H-15), 2.84 (H-15′), and 6.13 (H-10) and a carbon resonance at δ 172.4 (C-9) located a carbonyl α to the amine and identified this fragment as an *N*-acylated amino acid. The remaining carbon in this fragment was shown by HMBC correlations from proton resonances at δ 1.42 (Me-17), 3.09 (H-16), 1.65 (H-15), and 2.84 (H-15′) to have a chemical shift of δ 106.5, typical of calculated and reported values for trichlorinated methyl groups.[4] Thus, fragment A was identified as an *N*-propionyl trichloroleucine residue.

^{15}N-HMQC correlations between the ^{1}H resonances at δ 4.16 (H-4), 1.78 (H-3), and 1.97 (H-3′) and the ^{15}N resonance at δ −212.0 (*N*-8) demonstrated that one of the methine carbons at the terminus of this ^{1}H spin system was attached to a nitrogen atom. An HSQC correlation observed between the ^{1}H resonanace at δ 5.45 (H-18) and a carbon resonance at δ 78.7 (C-18) showed that the other terminal methine carbon in this ^{1}H spin system had a chemical shift typical of a dichlorinated methyl group,[3] leading to the assignment of the spin system to a dichloroleucine side chain. HMBC correlations between ^{1}H resonances at δ 1.78 (H-3), 1.97 (H-3′), 4.16 (H-4), 4.47 (H-6), and 2.79 (Me-20) and a carbon resonance at δ 179.0 (C-5) showed that the methine carbon (C-4) attached to nitogen was also linked to an alkene carbon (C-5) bearing a methyl ether. Additional HMBC correlations between the ^{1}H resonances at δ 4.16 (H-4) and 4.47 (H-6) and a carbon resonance at δ 169.1 (C-7) showed that the methoxy-bearing alkene carbon (C-5) and the olefinic methine carbon were part of an enolized and methylated β dicarbonyl substructure. An ^{15}N-HMQC correlation observed between the olefinic alkene resonance at δ 4.47 (H-6) and the ^{15}N resonance at δ −212.0 (*N*-8) demonstrated that the C-7 carbonyl was linked to *N*-8 via an amide bond to complete the ring required by the unsaturation number (Fragment B, Figure 1). Finally, an HMBC correlation observed between the methine resonance at δ 4.16 (H-4) and the carbonyl resonance at δ 172.4 (C-9) showed that Fragments A and B were linked via an amide bond, completing the constitution of sintokamide A (**1**).

It was not possible to determine the relative or absolute configuration of sintokamide A (**1**) from the NMR data. Fortunately, **1** gave crystals from MeOH that were suitable for X-ray diffraction analysis (Supporting Information). The ORTEP diagram for sintokamide A shown in Figure 2

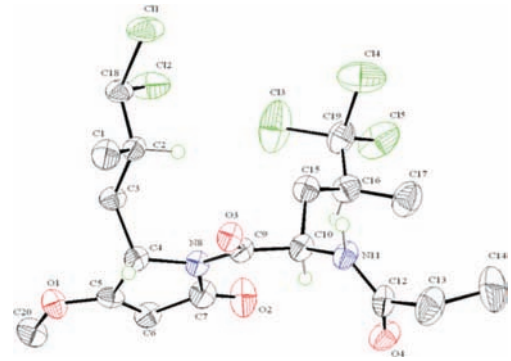

Figure 2. ORTEP drawing for sintokamide A (**1**).

confirmed the constitution proposed from the NMR analysis. Assignment of the absolute configuration 2*S*,4*S*,10*R*,16*S* was made possible by the anomalous dispersion from the five chlorine atoms and is based on the refined Flack parameter of 0.01(3).[5]

The structures of sintokamides B to E (**2**−**5**) differ from sintokamide A (**1**) simply in the degree of chlorination at Me-18 or Me-19. With the structure of sintokamide A (**1**) in hand, it was a routine exercise to assign constitutions to **2**−**5** from their spectroscpic data, and therefore, the details are not presented here (see Supporting Information for spectra and tabulated NMR data). It has been assumed that sintoka-

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mides B to E (**2**−**5**) have the same absolute configuration (2*S*,4*S*,10*R*,16*S*) as sintokamide A (**1**).

The morphology of LNCaP cells treated for 48 h with 10 μg/mL of **1** showed no obvious signs of toxicity, indicating that the inhibitory effect of sintokamide A (**1**) on AR activation observed in the screening assay was not simply due to general cytotoxicity. Reporter specificity has been observed for the AR. The PSA-luciferase reporter gene construct contains a natural promoter and enhancer region with several well-characterized AREs.[6] Sintokamide A (**1**) (5 μg/mL) blocked AR activity as measured using the PSA(6.1)-luciferase reporter (Figure 3A). To determine if **1** blocked transactivation of the AR NTD, LNCaP cells were transfected with the plasmids for the AR NTD-Gal4DBD chimera protein and the Gal4-luciferase reporter and pretreated for 1 h with **1** (5 μg/mL) prior to addition of forskolin (FSK, 50 μM) for an additional 24 h. Consistent with previous reports, FSK transactivated the AR NTD. Sintokamide A (**1**) reduced FSK-induced transactivation of the AR NTD to baseline levels (Figure 3B) demonstrating that **1** inhibited transactivation of the AR NTD.

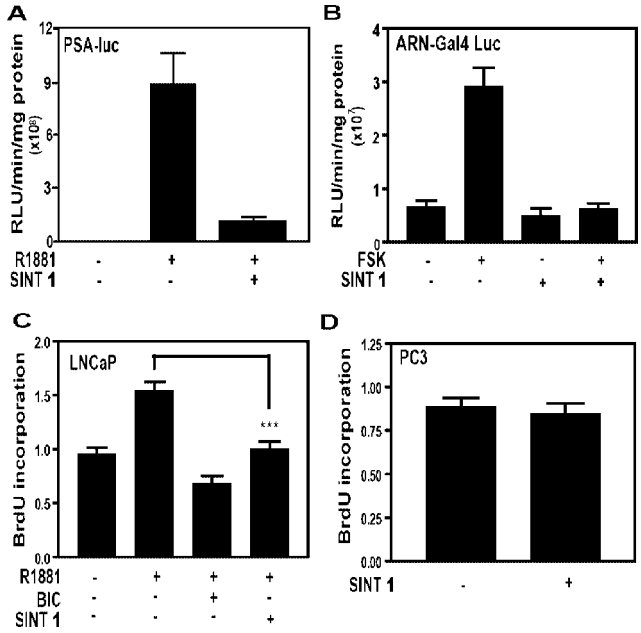

Figure 3. (A) Sintokamide A (**1**) (5 μg/mL) blocks AR activity as measured with the PSA-luciferase reporter. (B) **1** inhibits transactivation of the AR NTD stimulated by forskolin. (C) LNCaP cells treated with bicalutamide (BIC, 10 μM) or **1** (5 μg/mL) for 1 h before the addition of R1881 (0.1 nM) and incubated for 3 days. Cells were harvested and measured for BrdU incorporation. p = 0.0001 between **1** plus R1881 (column 4) to solely R1881-treated (column 2). (D) PC3 cells grown in the absence of serum for 1 day.

Sintokamide A (**1**) was found to be as effective as the control AR antagonist bicalutamide in blocking androgen-induced proliferation in androgen-sensitive LNCaP prostate cancer cells (Figure 3C). However, **1** did not block proliferation of PC3 human prostate cancer cells that do not express AR and thus do not rely on the AR for growth and survival (Figure 3D). These data are consistent with sintokamide A blocking AR signaling in androgen-sensitive prostate cancer cells.

Sintokamides A to E (**1**−**5**) are new members of a small family of chlorinated peptides that have been isolated from marine sponges,[2,4] nudibranchs,[7] and cyanobacteria.[8] Even though the sintokamides have been isolated from a sponge extract, the presence of a D-amino acid, chlorination of the leucine methyls, and an amino acid modified by extension with an acetate group[9] are all hallmarks of cyanobacterial metabolism, suggesting a microbial origin for the compounds.

Current therapies for prostate cancer target the C-terminus, ligand-binding domain of the AR and tend to fail presumably due to mutations such that these same antagonists become agonists. The sintokamides are the first small molecules known to selectively block transactivation of the N-terminus of the AR in prostate cancer cells. They show activity as novel antagonists to the AR making them promising experimental tools and drug leads for use in evaluating and developing a new approach to treating castration-recurrent prostate cancer.

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Supporting Information Available: Experimental details, tables of NMR asignments, and 1D and 2D NMR spectra for **1**−**5**. Materials and methods for the biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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