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ZHD-0501, a novel naturally occurring staurosporine analog from *Actinomadura* sp. 007

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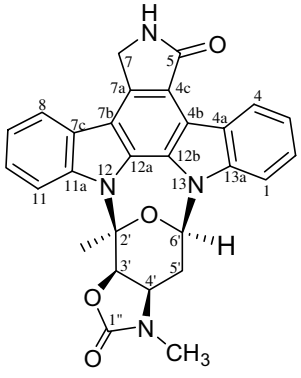
Abstract—ZHD-0501, a novel naturally occurring staurosporine analog showing anticancer activity in vitro, was isolated from the fermentation broth of a marine-derived *Actinomadura* sp. 007 through a bioassay-guided separation procedure and its structure elucidated by spectroscopic methods. ZHD-0501 provided the first example of staurosporin analog carrying a heterocycle fused to the pyran ring adopting a boat conformation with C-2′ and C-5′ as the bow and stern, which inhibited the proliferation of mammalian cancer A549, BEL-7402, HL60, P388, and tsFT210 cells.

Alkaloids with an indolocarbazole skeleton represented by the aglycone of staurosporine[1–3] come into being a very interesting class attracting a great attention[4–10] for their unusual structures and important biological activities. Structures of these compounds except for the non-glycosidic derivatives are all constructed from an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5(6*H*)-one skeleton and a sugar moiety connected by an unusual double *N*-glycosidic linkage or a single *N*-glycosidic linkage. Compounds with the double *N*-glycosidic linkage involve the *N*-pyranosidic derivatives[1–9,11–18] with staurosporine as the representative and the *N*-furanosidic derivatives[10,19,20] like K-252a,[20] while the single *N*-glycosidic derivatives are all *N*-pyranosidic as seen in K-252d.[20,21] All these types of alkaloids are naturally occurring[1–3,11–22] and a number of their analogs have been artificially synthesized.[4–10,23] On the other hand, X-ray analysis[2,3] and NMR studies[15–17,22] have demonstrated both solid state and solution conformations of the pyran ring in staurosporine and its analogs, that is, the chair conformation for the 4′-N free base and the boat conformation for the 4′-N protonated form, respectively. Herein, we report a novel natural staurosporine analog with an oxazolone ring fused to the pyran ring adopting a boat conformation with C-2′ and C-5′ as its bow and stern, which was produced by a marine-derived *Actinomadura* sp. 007 and designated as ZHD-0501 (**1**).

During our screening for new anticancer agents from microbial resources,[24–27] an actinomycete strain 007 isolated from a sea sediment sample collected in Jiaozhou



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Keywords: Indolocarbazole alkaloid; Staurosporines; ZHD-0501; Marine-derived actinomyces; *Actinomadura*; Anticancer activity.
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Bay, China, was found to produce metabolites showing strong in vitro biological activities using cancer cell cultures. This strain was identified as a species of the genus *Actinomadura* through a taxonomic study and from its metabolites, ZHD-0501 (**1**) was isolated through a bioassay-guided separation procedure. The *Actinomadura* sp. 007 was fermented in a liquid medium (glucose 2%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, beef extract 0.3%, corn slurry 0.3%, yeast extract 1%, soluble starch 1%, $CaCO_3$ 0.2% in artificial sea water, pH 7.0) on a rotary shaker under 120 rpm at 28 °C for 7 days. The whole fermentation broth (20 L) was filtered to obtain a mycelial cake, which was exhaustively extracted with EtOAc to afford an oily extract (4.8 g). This extract was separated by column chromatography over silica gel, Sephadex LH-20 and RP-18, followed by further purification using HPLC on a RP-18 semi-preparative column (eluent, CH_3CN–H_2O 4:6; flow rate, 4 mL/min; detective wave length, 288 nm; retention time, 15 min) to obtain **1** (15 mg).

Compound **1**, pale yellow crystals ($CHCl_3$–MeOH) with mp 283.4–285.5 °C, $[\alpha]_D^{20}$ +83.2 (*c* 0.10, MeOH), gave its protonated-molecular ion $[M+H]^+$ peak at *m*/*z* 479 in TOFMS. The molecular formula of **1** could be determined as $C_{28}H_{22}N_4O_4$ (20 unsaturations) by HRTOFMS (*m*/*z* 479.1704, calcd for $M+H^+$ 479.1719) and NMR data (Table 1). Its UV spectrum in MeOH gave a characteristic absorption curve with the absorption maxima at 232 (ε 19,613), 243 sh (19,020), 275 sh (19,720), 290 (33,212), 318 (9936), 332 (9636), 351 (6748), and 369 nm (6923), indicating the presence of an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5(6*H*)-one skeleton[16–20] as chromophore in **1**. The IR (KBr) spectrum gave absorption bands at 3377 (NH), 2924, 2835 (CH_2), 1739 (oxazolone CO), 1677 (lactam CO), 1589, 1458, 1399, 1321, 1275, 1229, 1030, and 751 cm^{-1}.

The ^1H and ^{13}C NMR data for **1** are summarized in Table 1. The NMR data for the aglycone of **1**, analyzed by DEPT and 2D NMR techniques, further confirmed the same indolocarbazole unit in **1** as in staurosporine.[16,21] Analysis of the PFG ^1H–^1H COSY and PFG HMQC spectra (Table 1) enabled us to elucidate the structural parts related to the proton spin systems in the indolocarbazole skeleton and the quaternary

Table 1. 600 MHz ^1H and 150 MHz ^{13}C NMR data for ZHD-0501 (**1**) in DMSO-d_6[a]

No.	δ_H (*J* in Hz)	COSY[b]	NOE's[c]	HMBC[d]	δ_C
1	7.79 br d (8.4)	H-2		C-2, C-3, C-4a	108.74 d
2	7.53 ddd (8.4, 7.6, 1.1)	H-1, H-3		C-1, C-3, C-4, C-13a	125.51 d
3	7.32 br t (7.6)	H-2, H-4		C-1, C-2, C-4, C-4a, C-13a[e]	119.62 d
4	9.25 br d (7.6)	H-3		C-1, C-2, C-3, C-4b, C-13a	125.79 d
4a	—				122.39 s
4b	—				115.81 s
4c	—				120.31 s
5	—				171.62 s
NH	8.71 br s			C-4b[e], C-4c, C-5, C-7, C-7a, C-7b[e]	—
7	5.03 d (17.2)	H-7 (δ4.99)		C-4c, C-5, C-7a, C-7b	45.51 t
	4.99 d (17.2)	H-7 (δ5.03)		C-4c, C-5, C-7a, C-7b	
7a	—				132.98 s
7b	—				115.44 s
7c	—				124.61 s
8	8.05 br d (7.4)	H-9		C-7b, C-10, C-11a	121.24 d
9	7.39 br t (7.4)	H-8, H-10		C-7c, C-8, C-11, C-11a[e]	120.98 d
10	7.52 ddd (8.5, 7.4, 1.1)	H-9, H-11		C-8, C-9, C-11, C-11a	124.98 d
11	8.07 br d (8.5)	H-11		C-7c, C-9, C-10	116.65 d
11a	—				140.36 s
12a	—				128.60 s
12b	—				124.61 s
13a	—				136.43 s
2′	—				92.52 s
3′	5.30 d (8.8)	H-4′	H-4′	C-2′, 2′-CH_3, C-4′, C-5′, C-1″	75.41 d
4′	4.34 ddd (12.0, 8.8, 5.3)	H-3′, H*a*-5′, H*e*-5′	H-3′, H-6′	C-2′, C-3′, C-1″	52.07 d
5′	H*a* 2.01 ddd (13.6, 12.0, 10.0)	H-4′, H*e*-5′		C-3′, C-4′, C-6′	28.75 t
	H*e* 2.93 ddd (13.6, 6.0, 5.3)	H-4′, H*a*-5′	H-4′, H*a*-5′, H-6′, N-CH_3	C-3′, C-4′, C-6′	
6′	6.97 dd (10.0, 6.0)	H*a*-5′, H*e*-5′	H-1, H-4′, H*e*-5′	C-2′, C-5′, C-12b, C-13a	79.18 d
2′-CH_3	2.03 s			C-2′, C-3′	29.56 q
N-CH_3	2.59 s			C-4′, C-1″	28.25 q
1″	—				155.66 s

[a] Signal assignments were based on the results of DEPT, PFG ^1H–^1H COSY, PFG HMQC, PFG HMBC, and difference NOE experiments.
[b] Numbers in the column indicate the protons that correlated with the proton on the line in the PFG ^1H–^1H COSY.
[c] Numbers in the column indicate the protons at which NOE's were detected in the difference NOE experiment under irradiation at the proton on the line.
[d] Numbers in the column indicate the carbons that showed HMBC correlations with the proton on the line in the PFG HMBC spectrum ($^{lr}J_{CH} = 8.3$ Hz).
[e] Weak but significant HMBC correlation through four-bonds was detected between the carbon and the proton on the line in the PFG HMBC spectrum.

carbons in the skeleton could be assigned unambiguously based on the related HMBC correlations (Table 1) from the PFG HMBC experiment. Structural parts related to the proton spin systems in the sugar moiety were also elucidated by the PFG ^{1}H–^{1}H COSY and PFG HMQC spectra (Table 1) and the methyl pyranose ring could be demonstrated by the HMBC correlations of 2′-CH_3/C-2′, 2′-CH_3/C-3′, and H-6′/C-2′. The HMBC correlations of H-3′/C-1″, H-4′/C-1″, N-CH_3/C-1″, and N-CH_3/C-4′ confirmed the *N*-methyl oxazolone ring fused to C-3′ and C-4′ in the sugar moiety. The carbons C-2′ and C-6′ in the sugar ring could be connected to N-12 and N-13 in the aglycone according to their chemical shifts[16,21] (Table 1) and the HMBC correlations between H-6′ and the carbons C-12b, C-13a.

The stereochemistry of **1** could be elucidated on the basis of the coupling constants (Table 1) of the protons on the pyran ring and the results of difference NOE experiments. In the difference NOE experiments, irradiation at H-4′ caused NOE's on H-3′ and H-6′ while irradiation at H*e*-5′ caused NOE on N-CH_3, indicating that H-3′, H-4′, and H-6′ were all *axial* in the same orientation and the protons H*e*-5′ and N-CH_3 were near in space. Inspection of the HGS molecular model indicated to satisfy the NOE's observed, the pyran ring in **1** should adopt a boat conformation with C-2′ and C-5′ as its bow and stern (Fig. 1), where N-12 and H*a*-5′ were 'bowsprit' atoms, the C–N bond between 6′- and 13-positions possessed an *equatorial*-orientation, and the N-CH_3 and H*e*-5′ groups were near parallel. This stereochemistry was well supported by the coupling constants (Table 1) of the protons on the pyran ring. Thus, the stereochemistry of **1** could be established as shown in Figure 1.

Although the aglycone of **1** is considered to be produced biosynthetically from two intact tryptophan units as demonstrated in staurosporine,[30] **1** is still of biosynthetic interest from its special sugar moiety, which seems likely to be biosynthesized from *N*-formyl-staurosporine[13] by cyclization between 3′-OCH_3 and *N*-formyl groups.

Compound **1** inhibited the proliferation of human cancer A549, BEL-7402, and HL60 cells and mouse leukemia P388 cells with the inhibition rates of 82.6%, 57.3%, 76.1%, and 62.2%, assay by SRB[28] (A549 and BEL-7402) or MTT[29] (HL60 and P388) methods at 1 μM, respectively. It also inhibited the proliferation of mouse cancer tsFT210 cells with the inhibition rates of 28.3% at 21 μM and 20.5% at 2.1 μM in the SRB assay. Flow cytometric analysis[28,29] indicated that **1** inhibited the cell cycle of tsFT210 cells mainly at the G2/M phase.

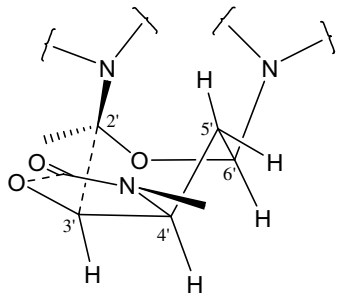

Figure 1. Conformation of pyran ring in **1**.

Although numerous staurosporine analogs have been known so far, **1** provides the first example of staurosporin analog carrying a heterocycle fused to the pyran ring adopting a boat conformation with C-2′ and C-5′ as the bow and stern, which inhibited the proliferation of mammalian cancer A549, BEL-7402, HL60, P388, and tsFT210 cells.

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