# Inhibition of Cytokine Production by Hymenialdisine Derivatives

Vasudha Sharma, Theresa A. Lansdell, Guangyi Jin, and Jetze J. Tepe*

*Department of Chemistry, Michigan State University, East Lansing, Michigan 48824*

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We describe herein the synthesis and biological activity of two indoloazepines that are structurally related to the marine sponge metabolite hymenialdisine. The natural product hymenialdisine was found to be a potent inhibitor of interleukin-2 ($IC_{50} = 2.4\ \mu M$) and tumor necrosis factor α ($IC_{50} = 1.4\ \mu M$) production. One of the hymenialdisine derived indoloazepines was found to also inhibit interleukin-2 ($IC_{50} = 3.5\ \mu M$) and tumor necrosis factor α ($IC_{50} = 8.2\ \mu M$) production.

Elevated levels of cytokines such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor α (TNF-α) have been linked to many disorders including Crohn's disease and psoriasis. In addition, these proinflammatory cytokines play an essential role in the pathogenesis of rheumatoid arthritis and osteoarthritis.[1−3] The inhibition of cytokines, in particular TNF-α, has been successful in several clinical trials for the treatment of rheumatoid arthritis.[4] However, variability in responses to these antiinflammatory drugs is found due to the complex network of alternative cytokine mediated pathways.[5] Inhibition of transcription factors that control the expression of several proinflammatory mediators such as the nuclear transcription factor NF-κB may overcome these problems and provide an alternative strategy in the treatment of these inflammatory disorders.[6] Because of its critical role in the regulation of inflammatory responses, NF-κB has become an increasingly significant therapeutic target in diseases such as asthma, rheumatoid arthritis, multiple sclerosis, and Alzheimer's disease.[7]

The marine sponge metabolite hymenialdisine (**1**, Figure 1) was originally isolated from the sponges *Axinella verrucosa* and *Acantella aurantiaca*, and its structure was established using X-ray crystallography.[8] Hymenialdisine was found to inhibit various proinflammatory cytokines such as IL-1, IL-6, IL-8, and nitric oxide in a variety of cell lines.[9,10] Investigation of the promising antiinflammatory properties of hymenialdisine revealed that the bromopyrrole alkaloid inhibits cytokine production through inhibition of the NF-κB signaling pathway. Gel shift analysis indicated that hymenialdisine selectively reduced NF-κB nuclear binding and not the DNA binding of other transcription factors such as C/EBP, AP-1, and SP1.[11] Recently, Meijer et al. reported that the potent NF-κB inhibitor hymenialdisine acts as a competitive nanomolar inhibitor of the cyclin-dependent kinases GSK-3β and CK1.[12] Crystallographic data portrayed the binding of the kinase inhibitor in the ATP binding pocket of the kinases. Considering the potential relationship between NF-κB activation and GSK-3, this might suggest a potential pathway for this kinase inhibitor.[13] In addition, Ireland and co-workers have recently also identified hymenialdisine as a very potent mitogen-activated protein kinase kinase-1 inhibitor with low-nanomolar $IC_{50}$ values.[14]

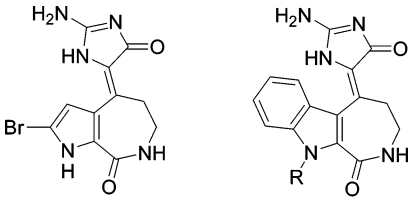


**Figure 1.** Structures of hymenialdisine and its indoloazepine derivatives **2** and **3**.

As part of our program to develop small-molecule inhibitors of NF-κB mediated gene transcription, we describe here the synthesis and biological evaluation of two hymenialdisine-derived indoloazepines: **2** and **3**. Compound **2** was shown to inhibit IL-2 and TNF-α production, whereas the methylated product **3** was depleted of any significant activity. The natural product **1** was found to inhibit IL-2 and TNF-α production as well, with similar $IC_{50}$ values.

## Synthesis and Biolgical Evaluation

The synthesis of the indol-aldisine or indoloazepine sketeton was achieved via a slightly modified route as reported in the synthesis of aldisine.[15,16] From the commercially available 2-indolecarboxylic acid, condensation with the ethyl ester of β-alanine in the presence of EDCI and DMAP provided the indole **4**. Methylation of the indole nitrogen with MeI and $K_2CO_3$ proceeded in near-quantitative yields to give the *N*-methylindole, which was used for the synthesis of **3**. Hydrolysis of esters **4** and **5** followed by the $P_2O_5/MeSO_3H$ mediated cyclization provided the key intermediate aldisine derivatives **8** and **9**. $TiCl_4$ mediated aldol condensation with the phenyloxazolone **12** provided the oxazolone successfully in 55% and 56% yield for **10** and **11**, respectively. Treatment of the oxazolone derivatives **10** and **11** with the *S*-benzylthiourea under basic conditions provided the final products **2** and **3** in modest yields (Scheme 1). Compound **3** was successfully crystallized

* To whom correspondence should be addressed. Phone: 517-355-9715, extension 147. Fax: 517-353-1793. E-mail: tepe@cem.msu.edu.

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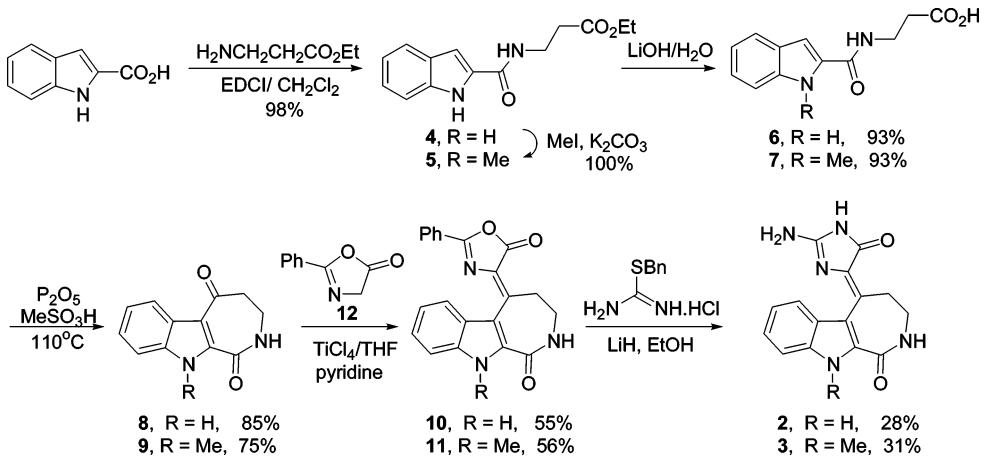
**Scheme 1.** Synthesis of **2** and **3**




from methanol, and its crystal structure is illustrated in Figure 2. Clearly indicated in this crystal structure

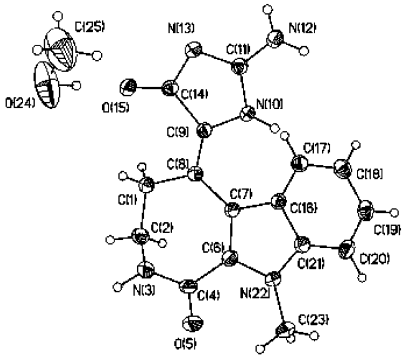


**Figure 2.** X-ray crystal structure of **3**·MeOH.

is the Z double bond at the C(8)−C(9) position of **3** as found in the natural product **1**.

Hymenialdisine and indoloazepines **2** and **3** were evaluated for their potential antiinflammatory activity by examining the transcriptional activity of NF-$\kappa$B in human Jurkat leukemia T-cells and THP-1 cells (human monocytic leukemia cell line). Exposure of human Jurkat leukemia T-cells to phorbol myristate acetate (PMA/PHA) and THP-1 cells to lipopolysaccharide (LPS) activates the NF-$\kappa$B mediated gene transcription of several proinflammatory cytokines, including IL-2, IL-6, IL-8, and TNF-α. The antioxidant and nonselective NF-$\kappa$B inhibitor pyrrolidinedithiocarbamate (PDTC) has been reported to repress activation of NF-$\kappa$B and was used as a control in all experiments. The effect of hymenialdisine and the indoloazepines **2** and **3** on NF-$\kappa$B's transcriptional activity was evaluated by measuring the level of IL-2 and TNF-α production using a competitive enzyme immunoassay (EIA). The inhibition of IL-2 was evaluated in Jurkat cells after PMA/PHA activation.[17] The inhibition of TNF-α was evaluated in THP-1 cells after LPS activation. NF-$\kappa$B mediated transcription of IL-2 production was examined by exposing the cells to PDTC or **1**−**3** 30 min prior to PMA activation. After 24 h, the cell-free supernatants were collected and subjected to EIA for the quantification of total IL-2 production. Hymenialdisine exhibited significant inhibition of IL-2 production with an $IC_{50}$ of 2.4 $\mu$M (Table 1). Treatment of the Jurkat cells with **2** at concentrations of 0.1−10 $\mu$M exhibited also a significant

**Table 1.** Inhibition of Cytokine (IL-2 and TNF-α) Production Measured by EIA for PDTC and **1**−**3**

| compd | IL-2 production $IC_{50}$ ($\mu$M) | TNF-α production $IC_{50}$ ($\mu$M)[a] |
|---|---|---|
| PDTC (control) | 5.12(±0.41) | 6.11(±0.53) |
| hymenialdisine **1** | 2.41(±0.71) | 1.36(±0.25) |
| **2** | 3.55(±0.09) | 8.16(±0.31) |
| **3** | >10 | >10 |

[a] Values are mean of two experiments. Standard deviation is given in parentheses.

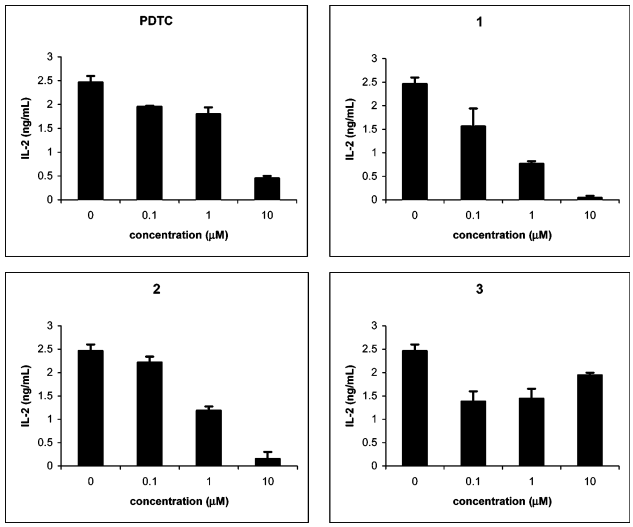



**Figure 3.** IL-2 production measured by EIA with PDTC and **1**−**3**.

dose−response inhibition of IL-2 production (Figure 3). Compound **2** exhibited an inhibition of IL-2 production with an $IC_{50}$ of 3.5 $\mu$M. Interestingly, blockage of the *N*-indole position with a methyl group (**3**) indicated no significant inhibition of IL-2 production (Figure 3, measured to 10 $\mu$M). This further supports the significance of the pyrrolic N−H moiety in potential hydrogen-bonding interactions.

We also examined **1**−**3** for their ability to inhibit TNF-α production in LPS stimulated THP-1 cells. Treatment of THP-1 cells with PDTC and **1**−**3** 30 min prior to LPS activation resulted in a significant inhibition of TNF-α production after a 3 h incubation period (Table 1). Hymenialdisine was found to be a potent inhibitor of TNF-α production with an $IC_{50}$ of 1.4 $\mu$M. Compound **2** was also found to inhibit TNF-α produc-

**Table 2.** Inhibition of Cell Growth with **1**−**3** in CEM Leukemia T Cells

| compd | inhibition of cell growth $GI_{50}$ ($\mu$M)[a] |
|---|---|
| PDTC (control) | N/T[b] |
| hymenialdisine **1** | 1.61(±0.62) |
| **2** | 1.73(±0.088) |
| **3** | 14.3(±2.41) |

[a] Values shown are average of two experiments. Standard deviation is given in parentheses. [b] N/T: not tested.

tion, albeit it was less potent than **1** ($IC_{50}$ = 8.2 $\mu$M). The methylated indoloazepine **3** was again depleted of any significant activity (tested to 10 $\mu$M, Table 1).

Inhibition of the DNA binding of NF-$\kappa$B by **1**−**3** was evaluated by examining the nuclear extracts of PMA/PHA activated Jurkat cells using gel electrophoresis (EMSA). Nuclear extracts were prepared from Jurkat cells that were treated with various concentrations of PDTC and **1**−**3** for 30 min before PMA/PHA stimulation for an additional 30 min. Compound **2** (5.0 $\mu$M) indicated approximately 49% inhibition of NF-$\kappa$B−DNA binding, compared to approximately 46% for hymenialdisine (5.0 $\mu$M) (data not shown). Compound **3**, however, failed to show any significant inhibition. Similar to the studies reported on hymenialdisine, no significant inhibition of DNA binding was observed when compounds **1**−**3** were tested for inhibition of DNA binding with the transcription factor AP-1 (data not shown).[11]

Hymenialdisine, **2**, and **3** were tested for their cytotoxicity and were found to exhibit significant inhibition of cell growth (Table 2). Cells were treated at different concentrations from 10 nM to 5 $\mu$M over a 48 h time period. Hymenialdisine and **2** indicated similar inhibition of cell growth with a $GI_{50}$ of 1.61 and 1.73 $\mu$M, respectively, whereas **3** indicated weaker inhibition of cell growth ($GI_{50}$ = 14.3 $\mu$M). The inhibition of cell growth with **1**−**3** could therefore account for some of the inhibition of IL-2 production seen in the Jurkat cells, where IL-2 production was measured 24 h after PMA stimulation. However, inhibition of TNF-α production was measured within 3 h after LPS stimulation and no significant inhibition of cell growth was apparent at this time interval.

## Conclusion

We have described herein the synthesis of a new indoloazepine inhibitor of the proinflammatory protein NF-$\kappa$B. Inhibition of the transcriptional activity of NF-$\kappa$B by indoloazepines **2** and **3** was evaluated by measuring IL-2 and TNF-α production in Jurkat and THP-1 cells. The free indoloazepine **2** and hymenialdisine exhibited potent inhibition of IL-2 and TNF-α production, whereas the *N*-methylindoloazepine **3** was depleted of any significant activity. Hymenialdisine and indoloazepine **2** were found to inhibit DNA binding by NF-$\kappa$B. Gel electrophoresis assays indicated that none of the compounds had any notable effect on the DNA binding of the transcription factor AP-1. Therefore, the indoloazepine **2** provides a new synthetic, readily available scaffold for further optimization for cytokine inhibition. The further clinical potential and details on the mode of action of this new class of indoloazepines is currently under investigation in our laboratory and will be reported shortly.

## Experimental Section

All commercial reagents were used without further purification. All solvents were reagent grade. THF was freshly distilled from sodium/benzophenone under nitrogen. $CH_2Cl_2$ was freshly distilled from $CaH_2$ under nitrogen. Column chromatography was carried out on silica gel 60 (230−400 mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. Proton and carbon NMR spectra were recorded on a Varian Gemini-300 spectrometer or a Varian VXR-500 spectrometer. High-resolution mass spectra and FAB mass spectra were obtained at the Mass Spectrometry Laboratory of the Michigan State University with a JEOL AX-505H and a JEOL HX-110 double-focusing mass spectrometer (JEOL USA, Peabody, MA), respectively.

**3-[(1*H*-Indole-2-carbonyl)amino]propionic Acid Ethyl Ester, 4.** To a mixture of 2-indolecarboxylic acid (5.01 g, 31.11 mmol) and 4-(dimethylamino)pyridine (6.44 g, 52.72 mmol) in 150 mL of anhydrous $CH_2Cl_2$ was added *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI·HCl) (7.13 g, 37.21 mmol) and $\beta$-alanine ethyl ester hydrochloride (5.71 g, 37.21 mmol) at 0 °C. The mixture then was stirred at 0 °C for 4 h and allowed to warm to room temperature for 20 h. The solution was washed with water (50 mL) and 10% HCl (50 mL). The reaction mixture was then dried over anhydrous $Na_2SO_4$, filtered, and concentrated to yield **4** as a white solid (7.95 g, 98%). The solid was recrystallized in methanol.

**3-[(1-Methyl-1*H*-indole-2-carbonyl)amino]propionic Acid Ethyl Ester, 5.** A solution of **4** (2.5 g, 9.57 mmol), $K_2CO_3$ (5.94 g, 43.06 mmol), and iodomethane (1.49 mL, 23.92 mmol) in acetonitrile (75 mL) was stirred at reflux for 24 h. The mixture was cooled and filtered, and the filtrate was evaporated in vacuo. The solid was then washed with 1 N HCl and extracted in $CH_2Cl_2$. The crude material was further purified by column chromatography on silica gel ($CH_2Cl_2$−EtOAc 3:1) to yield **5** (2.64 g) as white solid (100%). The product was recrystallized with dichloromethane/ether (1:1)

**3-[(1*H*-Indole-2-carbonyl)amino]propionic Acid, 6.** A mixture of **4** (6.1 g, 23.4 mmol) and $LiOH·H_2O$ (1.96 g, 46.7 mmol) was stirred at room temperature in ethanol (300 mL) for 20 h, and the ethanol was evaporated to dryness in vacuo. The residue was dissolved in water (50 mL), and the solution was acidified to pH 3 with concentrated HCl to form a white solid. The mixture was allowed to stand at 0 °C for 30 min, and the product was isolated by filtration. The solid was washed with water and recrystallized with methanol to afford **6** (5.03 g, 93%).

**3-[(1-Methyl-1*H*-indole-2-carbonyl)amino]propionic Acid, 7.** The procedure is similar as for **6** to provide **7** from **5** (2.16 g, 93%). The product was recrystallized in methanol.

**3,4-Dihydro-2*H*,10*H*-azepino[3,4-*b*]indole-1,5-dione, 8.** Compound **6** (2 g, 8.6 mmol) was added to a clear solution of $P_2O_5$ (1.33 g, 9.44 mmol) in $MeSO_3H$ (70 mL) at 60 °C. The mixture was heated to 110 °C for 1.5 h and then cooled to room temperature. The mixture was poured into ice−water and stirred for 30 min. The buff-colored solid was then filtered and dissolved in acetone (250 mL) and filtered again, and the filtrate was concentrated to give a light-brown solid that was further purified by column chromatography (20% acetone in $CH_2Cl_2$) on silica gel to yield **8** (1.56 g, 85%).

**10-Methyl-3,4-dihydro-2*H*,10*H*-azepino[3,4-*b*]indole-1,5-dione, 9.** The procedure is similar as for **8** to provide **9** from **7** (1.3 g, 75%). The product was recrystallized in methanol.

**5-(5-Oxo-2-phenyloxazol-4-ylidene)-3,4,5,10-tetrahydro-2*H*-azepino[3,4-*b*]indol-1-one, 10.** A solution of $TiCl_4$ (2.04 mL, 18.68 mmol) in $CH_2Cl_2$ (21 mL) was added into THF (65 mL) at −10 °C. Compound **8** (1 g, 4.67 mmol) and 2-phenylazlactone (1.50 g, 9.34 mmol) were subsequently added and allowed to stir at 0 °C for 20 min. Pyridine (2.09 mL, 37.36 mmol) was then added over a 30 min period. The reaction mixture was stirred at 0 °C for an additional 2 h and then allowed to stir overnight at room temperature. $NH_4Cl$ (80 mL,

saturated solution in water) was added, and the mixture was stirred for 10 min and subsequently extracted with ethyl acetate (3×). The organic extracts were combined dried, filtered, and concentrated, and the residue was purified using column chromatography on silica gel (EtOAc/hexane 7:3) to yield **10** (0.91 g, 55%) as a reddish-yellow solid.

**10-Methyl-5-(5-oxo-2-phenyloxazol-4-ylidene)-3,4,5,10-tetrahydro-2*H*-azepino[3,4-b]indol-1-one, 11.** The procedure is similar as for **10** to provide **11** from **9** (0.73 g, 56%) as a yellow solid.

**5-(2-Amino-5-oxo-1,5-dihydroimidazol-4-ylidene)-3,4,5,10-tetrahydro-2*H*-azepino[3,4-*b*]indol-1-one, 2.** LiH (16 mg, 2 mmol) was dissolved in ethanol (60 mL), and to this solution *S*-benzylisothiouronium chloride (405 mg, 2 mmol) was added. The mixture was stirred until the solution became clear. Compound **10** (150 mg, 0.4 mmol) was added, and the mixture was heated at reflux for 48 h. After the mixture cooled, the solvent was evaporated in vacuo. Ethanol (20 mL) was added and subsequently evaporated three times. Ethanol (5 mL) was added again, and the mixture was refluxed for 3 h. The solvent was evaporated in a vacuum, 1 N $HCl_{aq}$ (15 mL) was added, the mixture was extracted with *n*-butanol (3 × 15 mL), the extracts were washed with brine (3 × 10 mL), dried, and concentrated, and the product was purified twice by column chromatography on silica gel ($CH_2Cl_2$/MeOH, 4:1) to yield **2** (35 mg, 28%) as a light-yellow solid. **2**·HCl: Anal. ($C_{15}H_{14}N_5O_2Cl$) H, N. C: calcd, 54.30; found, 54.56.

**5-(2-Amino-5-oxo-1,5-dihydroimidazol-4-ylidene)-10-methyl-3,4,5,10-tetrahydro-2*H*-azepino[3,4-*b*]indol-1-one, 3.** The procedure is similar as for **2** to provide **3** from **11**. Product **3** was purified by column chromatography on silica gel, eluting with 85:15 $CH_2Cl_2$/MeOH followed by 88:12 $CH_2Cl_2$/MeOH to yield **3** as a light-yellow solid (96 mg, 31%). Anal. ($C_{16}H_{15}N_5O_2$) C, H, N. **3**·HCl: Anal. ($C_{16}H_{16}N_5O_2Cl$) C, H, N.

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**Supporting Information Available:** Experimental procedures for ELISA IL-2, ELISA TNF-α, inhibition of cell growth, $^1$H NMR, $^{13}$C NMR, IR, MS, and EA data for **2**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Gerard, C.; Rollins, B. J. Chemokines and disease. *Nat. Immunol.* **2001**, *2*, 108−115.
(2) Harris, E. D., Jr. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N. Engl. J. Med.* **1990**, *322*, 1277−1289.
(3) Baeuerle, P. A.; Henkel, T. Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* **1994**, *12*, 141−179.
(4) Moreland, L. W. Inhibitors of tumor necrosis factor for rheumatoid arthritis. *J. Rheumatol.* **1999**, *26* (Suppl. 57), 7−15.
(5) Handel, M. L.; Nguyen, L. Q.; Lehmann, T. P. Inhibition of transcription factors by anti-inflammatory and anti-rheumatic drugs: can variability in response be overcome? *Clin. Exp. Pharmacol. Physiol.* **2000**, *27*, 139−144.
(6) Tak, P. P.; Firestein, G. S. NF-kappaB: a key role in inflammatory diseases. *J. Clin. Invest.* **2001**, *107*, 7−11.
(7) Yamamoto, Y.; Gaynor, R. B. Role of the NF-kappaB pathway in the pathogenesis of human disease states. *Curr. Mol. Med.* **2001**, *1*, 287−296.
(8) Cimino, G.; De Rosa, S.; De Stefano, D.; Mazzarella, L.; Puliti, R.; Sodano, G. Isolation and x-ray crystal structure of a novel bromo-compound from two marine sponges. *Tetrahedron Lett.* **1982**, *23*, 767−768.
(9) Badger, A. M.; Cook, M. N.; Swift, B. A.; Newman-Tarr, T. M.; Gowen, M.; Lark, M. Inhibition of interleukin-1-induced proteoglycan degradation and nitric oxide production in bovine articular cartilage/chondrocyte cultures by the natural product, hymenialdisine. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 587−593.
(10) Breton, J. J.; Chabot-Fletcher, M. C. The natural product hymenialdisine inhibits interleukin-8 production in U937 cells by inhibition of nuclear factor-kappaB. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 459−466.
(11) Roshak, A.; Jackson, J. R.; Chabot-Fletcher, M.; Marshall, L. A. Inhibition of NFkappaB-mediated interleukin-1beta-stimulated prostaglandin E2 formation by the marine natural product hymenialdisine. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 955−961.
(12) Meijer, L.; Thunnissen, A. M.; White, A. W.; Garnier, M.; Nikolic, M.; Tsai, L. H.; Walter, J.; Cleverley, K. E.; Salinas, P. C.; Wu, Y. Z.; Biernat, J.; Mandelkow, E. M.; Kim, S. H.; Pettit, G. R. Inhibition of cyclin-dependent kinases, GSK-3beta and CK1 by hymenialdisine, a marine sponge constituent. *Chem. Biol.* **2000**, *7*, 51−63.
(13) Ali, A.; Hoeflich, K. P.; Woodgett, J. R. Glycogen synthase kinase-3: properties, functions, and regulation. *Chem. Rev.* **2001**, *101*, 2527−2540.
(14) Tasdemir, D.; Mallon, R.; Greenstein, M.; Feldberg, L. R.; Kim, S. C.; Collins, K.; Wojciechowicz, D.; Mangalindan, G. C.; Concepcion, G. P.; Harper, M. K.; Ireland, C. M. Aldisine alkaloids from the Philippine sponge Stylissa massa are potent inhibitors of mitogen-activated protein kinase kinase-1 (MEK-1). *J. Med. Chem.* **2002**, *45*, 529−532.
(15) Annoura, H.; Tatsuoka, T. Total synthesis of hymenialdisine and debromohymenialdisine: stereospecific construction of the 2-amino-4-oxo2-imidazolin-5(Z)-disubstituted ylidene ring system. *Tetrahedron Lett.* **1995**, *36*, 413−416.
(16) Xu, Y, Y.; Yakushijin, K.; Horne, D. A. Synthesis of C(11)N(5) Marine Sponge Alkaloids: (±)-Hymenin, Stevensine, Hymenialdisine, and Debromohymenialdisine. *J. Org. Chem.* **1997**, *62*, 456−464.
(17) Mahon, T. M.; O'Neill, L. A. J. Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-kappa B activation in T lymphocytes. *J. Biol. Chem.* **1995**, *270*, 28557−28564.

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