Organic LETTERS

Letter

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(±)-Aspongamide A, an *N*-Acetyldopamine Trimer Isolated from the Insect *Aspongopus chinensis*, Is an Inhibitor of p-Smad3

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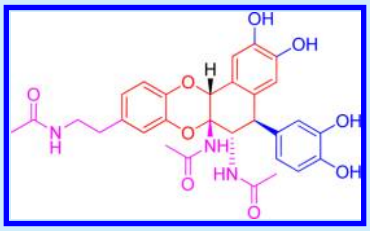
Supporting Information

ABSTRACT: (±)-Aspongamide A (**1**), an unusual trimer of *N*-acetyldopamine (NADA) bearing a novel tetrahydrobenzo[*a*]dibenzo[*b*,*e*][1,4]dioxine structure, and a pair of NADA dimeric enantiomers (**2**) were isolated from *Aspongopus chinensis*. The structures of compounds **1** and **2** were assigned using spectroscopic methods. Compound **1** was found to be an inhibitor of Smad3 phosphorylation in transforming growth factor-β1 (TGF-β1) induced rat renal proximal tubular cells and suppressed extracellular matrix expression in mesangial cells under diabetic conditions.



Chronic kidney disease (CKD) is characterized by a progressive loss in renal function over a period of months or years. The incidence of CKD is growing worldwide owing to the prevalence of diabetes, which is one of the major causes of this disease. Unfortunately, no specific treatment that slows the progression of CKD has been developed thus far, and although some potential drugs are in the development process, their efficacies have not yet been demonstrated. CKD can lead to end-stage renal disease, where patients require dialysis or renal replacement therapy for survival.[1] Moreover, patients afflicted with CKD tend to develop cardiovascular disease, which then becomes the most common cause of death rather than renal failure.[2] Owing to the seriousness of CKD, an urgent need exists for the development of efficacious drugs for its treatment.

It is known that a complex set of factors, including transforming growth factor-β (TGF-β), advanced glycation end-products (AGEs), production of high glucose levels, and angiotension II (Ang II), are involved in CKD. Among these, TGF-β1 has been shown to play a significant pathogenic role in CKD,[3,4] exerting its cellular effects via either Smad-dependent or independent pathways. Smad signaling is considered to be the major mechanism by which TGF-β1 regulates CKD.[5] Among the Smads, which play diverse roles in CKD, Smad4 is an unsuitable therapeutic target, and the activation of Smad2 and Smad7 has a beneficial effect on CKD because both are renoprotective. In contrast, Smad3 is a key mediator of TGF-β/Smad signaling, and its over-phosphorylation plays an unequivocal role in promoting the pathogenesis of CKD.[5] Therefore, strategies that focus on the inhibition of TGF-β/Smad3 signaling should be effective in developing new therapeutic approaches for the treatment of CKD. To our knowledge, one small molecule inhibitor of this signaling process, namely SIS3, has been commercialized as a tool drug.[6] Thus, investigations in our laboratory in recent years have focused on the discovery of inhibitors of Smad3 phosphorylation.

Insects are the most diverse and abundant of all terrestrial animals. While the crucial role that insects play in the ecosystem has received great attention, studies of the chemical defense mechanisms used by insects began only in the late 1950s. Since the inception of these efforts, several structurally interesting small molecules, which are utilized as defensive substances, have been discovered.[7,8] However, in contrast to efforts exploring their species diversity and richness, as well as their peptide components, much less attention has been given to chemical substances that insects employ to combat natural enemies.[8] To even a greater extent, the potential roles that insect derived substances might play as lead compounds in drug discovery efforts has been largely ignored.

Insects are utilized as medicines or foods in the world, especially in China. The insect, *Aspongopus chinensis* Dallas (Pentatomidae), known as Jiu-Xiang-Chong, is an endemic food found in southern China. Owing to its effects on warming the stomach, relieving pain and restoring the kidney Yang, the dried body of *A. chinensis* is a common traditional Chinese medicine utilized to treat pain, poor digestion, and kidney diseases.[9] Recently, a new oxazole derivative has been isolated from this insect.[9]

During the investigation described below, which focused on the discovery of *A. chinensis* derived natural products that are active against CKD, we isolated and characterized the previously unknown substances (±)-aspongamide A (**1**), an

Received: November 24, 2013
Published: January 2, 2014

dx.doi.org/10.1021/ol403409v | *Org. Lett.* 2014, 16, 532–535

N-acetyldopamine (NADA) trimer that has a unique tetrahydrobenzo[*a*]dibenzo[*b*,*e*][1,4]dioxine structural core, and (−)-**2**, together with the known (+)-**2** (Supporting Information). Importantly, the results of an evaluation of their biological properties showed that **1** is an inhibitor of diabetic nephropathy, using high-glucose-induced mesangial cells, and against renal fibrosis in TGF-β1 induced rat renal proximal tubular cells, whereas the effects of **2** are almost opposite those of **1**. Observations made in this effort are described below.

Aspongamide A,[10] obtained as a white solid, has the molecular formula $C_{30}H_{31}N_3O_9$ as deduced from its HREIMS-determined exact mass (*m*/*z* 577.2045 $[M]^+$, calcd for 577.2060) and 17 degrees of unsaturation arising from analysis of its ^{13}C and DEPT NMR spectra. The ^1H NMR spectrum of compound **1** (Table 1) contains two ABX proton

Table 1. ^1H (600 MHz) and ^{13}C NMR (150 MHz) Data of 1 in Methanol-d_4 (δ, ppm)

position	^{13}C NMR ppm (mult)	^1H NMR ppm (mult, *J* in Hz)
1	134.0 s	
2	118.4 d	6.75 (d, 1.9)
3	142.1 s	
4	140.8 s	
5	118.8 d	6.89 (d, 8.4)
6	123.7 d	6.72 (dd, 8.4, 1.9)
7	35.8 t	2.67 (t, 7.2)
8	42.1 t	3.30 (overlap)
9	173.4 s	
10	23.4 q	1.83 (s)
1′	125.8 s	
2′	113.6 d	6.86 (s)
3′	146.2 s	
4′	145.6 s	
5′	116.8 d	6.15 (s)
6′	131.0 s	
7′	70.9 d	6.61 (overlap)
8′	85.8 s	
9′[a]	173.3 s	
10′[b]	22.5 q	1.87 (s)
1″	135.8 s	
2″	117.3 d	6.61 (overlap)
3″	145.3 s	
4″	146.3 s	
5″	115.8 d	6.69 (d, 8.4)
6″	122.4 d	6.60 (dd, 8.4, 1.9)
7″	49.5 d	3.97 (d, 10.8)
8″	56.2 d	5.02 (d, 10.8)
9″[a]	173.0 s	
10″[b]	22.3 q	1.88 (s)
8-*NH*[c]		7.96 (brt, 5.6)
8′-*NH*[c]		7.99 (s)
8″-*NH*[c]		7.90 (d, 9.4)

[a]The signals with the same signs may be interchanged. [b]The signals with the same signs may be interchanged. [c]Recorded in DMSO-d_6.

coupling patterns {(δ_H 6.75, d, *J* = 1.9 Hz, H-2; δ_H 6.89, d, *J* = 8.4 Hz, H-5; δ_H 6.72, dd, *J* = 8.4, 1.9 Hz, H-6), (δ_H 6.61, overlap, H-2″; δ_H 6.69, d, *J* = 8.4 Hz, H-5″; δ_H 6.60, dd, *J* = 8.4, 1.9 Hz, H-6″)}, two olefinic singlet resonances (δ_H 6.86, s, H-2′; δ_H 6.15, s, H-5′), three methyl singlets, along with two methylene and three methine resonances in the aliphatic region. The ^{13}C and DEPT NMR spectra (Table 1) show that this substance possesses 30 carbons comprised of three methyls, two methylenes, eleven methines (eight aromatic and/or olefinic), and fourteen quaternary carbons (three amide carbonyl, ten aromatic and/or olefinic including six oxygenated, and a downfield acetal carbon). Analysis of the spectroscopic data along with the recognition that *N*-acetyldopamine (NADA) polymers have been isolated from different insects (see below),[9,11] led to the proposal that compound **1** is likely a dopamine derivative consisting of three NADA units. In related studies, we have detected dimeric NADA derivatives in beetles such as *Blaps japanensis* and *Catharsius molossus* L. (dung beetle). The presence of three NADA units in compound **1** was

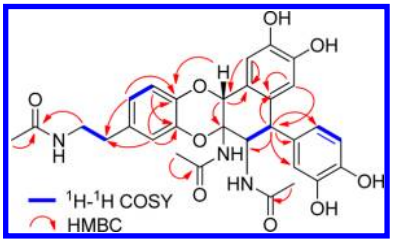
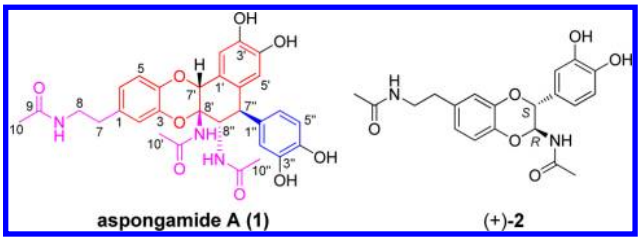


Figure 1. Key $^1H-^1H$ COSY and HMBC correlations for (±)-**1**.

supported by the following 2D NMR data (Figure 1): (1) $^1H-^1H$ COSY correlations of H-5/H-6 and H-7/H-8, HMBC correlations of H-10, H-8/C-9, H-2, H-6/C-4 (δ_C 140.8) and C-7, H-2, H-5/C-3 (δ_C 142.1); (2) HMBC correlations of H-2′/C-7′, H-7′/C-8′, H-2′/C-3′ (δ_C 146.2) and C-4′ (δ_C 145.6); (3) $^1H-^1H$ COSY correlations of H-5″/H-6″ and H-7″/H-8″, HMBC correlations of H-10″/C-9″, H-7″/C-1″, C-2″, and C-6″, H-2″/C-3″ (δ_C 145.3) and C-4″ (δ_C 146.3). Placement of these NADA units within the structure of **1** was mainly carried out by using the results of HMBC experiments. The fact that compound **1** bears a benzodioxane motif like that present in **2**, is supported by observations of HMBC correlations of H-7′/C-4 and C-8′, and the diagnostic chemical shift of C-8′ (δ_C 85.8). Apart from three benzene rings, one dioxane ring and three amide carbonyl groups, which together account for 16 degrees of unsaturation, the structure of **1** has an additional ring. The HMBC correlations between H-7″/C-6′ and H-5′/C-7″ suggest that C-6′ and C-7″ are connected and that C-8′ is connected to C-8″, which results in the formation of a six-membered ring. Although the key HMBC correlations of H-7′/C-8″, or H-8″/C-7′ or C-8′ were not detected, the observed ROESY correlation of H-8″/8′-*NH* provide sound evidence for the above proposal. Normally, the *N*-acetylamino-2-ethyl group is positioned at C-1 or C-6, but the observed HMBC correlations of H-2/C-3 and C-7, H-6/C-4 and C-7, and H-7′/C-4 suggest that this moiety is positioned at C-1.



The relative configurations of the four chiral centers present in the structure of **1** were assigned by employing ROESY

dx.doi.org/10.1021/ol403409v | *Org. Lett.* 2014, 16, 532−535

experiments with a DMSO-d_6 solution (Figure 2). The existence of ROESY correlations of H-7″/8″-*NH* and H-8″/H-2″ indicate that H-7″ and H-8″ are *trans*-disposed. Likewise, the ROESY cross peaks of H-7′/8′-*NH* and H-8″, and 8′-*NH*/H-8″ suggested that these protons have *cis* relationships. The lack of optical activity suggests that compound **1** was isolated as a racemate. Attempts to separate the enantiomers of **1** using different chiral phase columns were not successful.

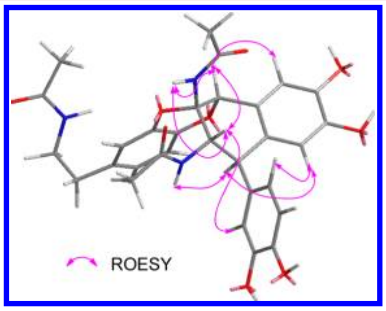


Figure 2. Important ROESY correlations of (±)-**1**.

Compound **2** was isolated from this species as a racemate. Racemic (±)-**2** was resolved by chiral-phase HPLC to afford (+)-**2** and (−)-**2**. We noted that (+)-**2** has been previously isolated from the cast-off shell of the cicada of *Cryptotympana* sp. However, we found that its absolute configuration was wrongly assigned.[12] Herein, the absolute configuration of (−)-**2** was assigned by computational methods which accordingly revised the conclusion in the literature (Supporting Information).

NADA derivatives are products of enzyme catalyzed reactions in the cuticle.[13] Metabolism of NADA generates a highly reactive 1,2-dehydro-NADA *o*-quinone species, which rapidly condenses with *o*-diphenols to form dimeric, trimeric, or higher oligomeric NADA derivatives containing one or more benzodioxan moieties.[13] (±)-Aspongamide A is composed of three NADA units within one benzodioxan motif. As such, compound **1** is probably an intermediate or a byproduct in sclerotization reactions generated by a pathway that involves a new NADA polymerization pattern, indicating that polymerization can occur at positions that are not *o*-diphenol sites. A plausible biosynthetic pathway for the production of compound **1** is presented in Scheme 1 (Supporting Information).

A. chinensis has been used for the treatment of kidney diseases. Thus, a study was purposely conducted to determine if the substances isolated from *A. chinensis* are renoprotective. Interestingly, we observed that the unusual trimeric NADA derivative **1** promotes a significant decrease in Smad3 expression and phosphorylation of Smad3 in rat renal proximal tubular cells (Figure 3), indicating its high possibility of antifibrosis activity. In addition, pretreatment of high-glucose-induced mesangial cells with compound **1** leads to attenuation of collagen IV, fibronectin, and IL-6 secretion in a dose- and time-dependent manner (Figures S13−S15, Supporting Information). As stated above, because TGF-β/Smad signaling dominates in the pathogenesis of CKD and we have found the inhibitory effect of compound **1** on Smad3 phosphorylation in rat renal proximal tubular cells, it is tentatively believed that the inhibitory effects of **1** in high-glucose-induced mesangial cells were through a TGF-β/Smad pathway.

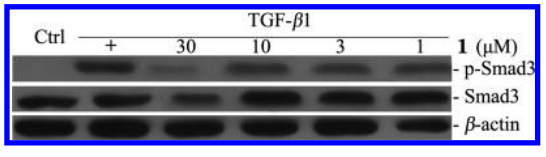


Figure 3. Compound **1** blocks TGF-β1-mediated Smad3 phosphorylation in a dose-dependent manner. NRK 52E cells were treated with TGF-β1 (10 ng/mL) for 1 h in the absence or presence of different doses of compound **1** as indicated. Ctrl: untreated cells.

In contrast to compound **1**, the dimeric NADA derivatives (+)-**2** and (−)-**2** were observed to cause an increase in collagen I and α-smooth muscle actin (α-SMA) expression in a dose- and time-dependent manner (Figures 4 and 5). The fact that these increases are hallmarks of fibrosis indicates that compound **2** may be beneficial for restoring skin after wounds because collagen I and α-SMA are implicated in the process of fibrosis and wound healing. But this suggestion needs to be further confirmed by using skin-related cells. Actually, NADA derivatives were also reported to be related to insect cuticular sclerotization.[14]

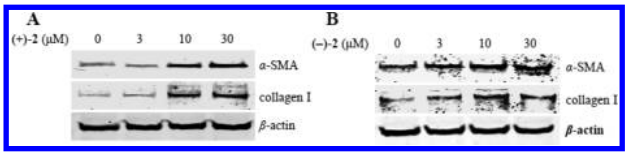


Figure 4. Compound (+)-**2** (A) or (−)-**2** (B) upregulates α-SMA and collagen I in a dose-dependent manner. NRK 52E cells were treated with (+)-**2** or (−)-**2** for 48 h at doses of 3, 10, and 30 μM as indicated. Cell lysates were immunoblotted with antibodies against α-SMA and collagen I. Data are expressed as mean ± SD. ANOVA, *p < 0.01 vs control. Ctrl: untreated cells.

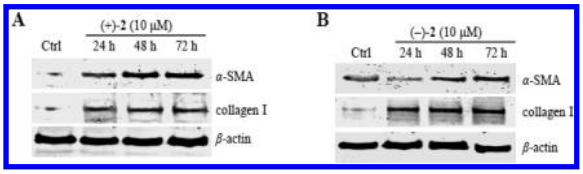


Figure 5. Compound (+)-**2** (A) or (−)-**2** (B) upregulates α-SMA and collagen I in a time-dependent manner. NRK 52E cells were treated with (+)-**2** or (−)-**2** at the dose of 10 μM for 24, 48, and 72 h as indicated. Western blot analyses were used to test the protein expression of α-SMA and collagen I. Data are expressed as mean ± SD. ANOVA, *p < 0.01 vs control. Ctrl: untreated cells.

Relatively few natural products (less than 1%) exist as racemic mixtures within the biosphere.[15] In general, insects should adopt economic ways to synthesize useful substances needed to maintain their physical functions, fit into their natural habitats, and combat natural enemies. However, we noted that NADA oligomers normally occur as racemates in the insects that we studied such as *A. chinensis*, *B. japanensis*, and *C. molossus*. This common phenomenon allows us to tentatively hypothesize that these oligomers may simply be the early-stage intermediates of random oxidation by enzyme prior to formation of high-molecular weight melanin-like molecules. In addition, we found that both (+)-**2** and (−)-**2** have no effect on phosphorylation of Smad2 or Smad3 (Supporting Information), indicating that other pathways are involved in collagen I and α-SMA expression. Finally, the results of assays show that compounds **1** and **2** are not cytotoxic.

dx.doi.org/10.1021/ol403409v | *Org. Lett.* 2014, 16, 532−535

In conclusion, the combined findings suggest that compound **1** is an ihibitor of p-Smad3 and suppresses extracellular matrix expression in mesangial cells under diabetic conditions. The effects of compound **2** are almost opposite those of **1**, revealing an interesting dual-directional regulation in cultured cell lines. Further studies of the biological function of compound **1** are expected.

■ ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR and MS spectra, detailed isolation procedures, bioassay methods, NMR data of **2**, computational methods and CD of **2**, partial biological data, and a plausible biosynthetic pathway for **1**. This material is available free of charge via Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported financially by grants from the National Natural Science Foundation of China (21272241; 21172223) and the Open Research Fund of State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources.

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(10) Aspongamide A (**1**): yellowish solid; $[\alpha]^{24}_{D}$ 0 (*c* 0.03, MeOH); UV (MeOH) λ_{max} 283 (3.77), 204 (4.67) nm; ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; ESIMS (positive) *m*/*z* 600 $[M + Na]^+$; HREIMS *m*/*z* 577.2045 $[M]^+$ (calcd for $C_{30}H_{31}N_3O_9$ 577.2060).
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