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Aspernigrins with anti-HIV-1 activities from the marine-derived fungus *Aspergillus niger* SCSIO Jcsw6F30

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ABSTRACT

Two new 2-benzylpyridin-4-one containing metabolites, aspernigrins C (**3**) and D (**4**), together with six known compounds (**1**, **2**, and **5**–**8**), were isolated from the marine-derived fungus *Aspergillus niger* SCSIO Jcsw6F30. The structures of the new compounds were determined by NMR, MS, and optical rotation analyses. All the isolated compounds were evaluated for their inhibitory activities against infection with HIV-1 SF162 in TZM-bl cells. Malformin C (**5**) showed the strongest anti-HIV-1 activity with IC_{50} of 1.4 ± 0.06 μM (selectivity index, 11.4), meanwhile aspernigrin C (**3**) also exhibited potent activity with IC_{50} of 4.7 ± 0.4 μM (selectivity index, 7.5).

Despite the success of current antiretroviral treatment (ART), infections with the human immunodeficiency virus 1 (HIV-1) remain a global threat to public health with more than 33 million infected individuals and 2.3 million new infections in 2012. Existence of several shortcomings of the current ART, including the emergence of resistant virus, severe side effects and the high cost, has posed an urgent need for the discovery and development of potent anti-HIV-1 drugs with novel modes of action.[1] Marine organisms have been proven to be excellent sources of biologically active compounds against HIV-1. Discovery of anti-HIV-1agents, especially with novel modes of action, from marine organisms is becoming more attractive and promising.[2]

During our continuous chemical study of marine-derived fungi, four 2-benzylpyridin-4-one-containing metabolites (**1**–**4**), including two structurally new aspernigrins C (**3**) and D (**4**), and other four known compounds (**5**–**8**) were isolated from the marine-derived black aspergilli, *Aspergillus niger* SCSIO Jcsw6F30 (Fig. 1). All of the compounds we obtained were tested for their inhibitory activities against chemokine receptor subtype 5 (CCR5) tropic HIV-1 SF162 infection. Herein, we describe the isolation, structural elucidation and biological evaluations of these compounds.

The fungal strain *A. niger* SCSIO Jcsw6F30[3] was cultured on Medium B agar plates and then incubated for 7 days.[4] The crude extract (10.5 g) was subjected to a silica gel column chromatography (CC) and the fractions were purified by semi-preparative HPLC and repeated silica gel CC, to obtain compounds **1**–**8** (Fig. 1).[5]

Compounds **1** and **2** were identified as aspernigrins A and B by comparison of their NMR, MS and optical rotation (OR) data with those available in the literature.[6,7] The structures of aspernigrins A and B were identified as 2-pyridone substructure initially,[6] and then revised to be 4-pyridone.[7,8] The OR data of **2** ($[\alpha]_D^{20}$ +38.5, *c* 0.1, DMSO) suggests it has the same *S*-configuration (C-7′) with aspernigrin B in the reference ($[\alpha]_D^{20}$ +37.8, *c* 0.5, DMSO).[6]

Aspernigrin C (**3**)[9] was suggested to be an *N*-acylcarboxyamide derivate, like the structures of pestalamide B[10] and nygerone A,[11] connected by 2-methylsuccinic acid moiety to the carboxamide of aspernigrin B (**2**), by HR-ESI-MS and NMR analysis. The planer structure of aspernigrin C (**3**), together with some key HMBC correlations, was showed in Figure 2 as a new compound. The structure of aspernigrin C (**3**) could be considered as the establishment of three partial structures (fragments A–C) showed in Figure 2. Fragments A + B consist the structure of aspernigrin B (**2**) we obtained, while B + C make up the structure of pestalamide B, which is a common product of black aspergilli strains.[12] Aspernigrin B (**2a**) and 2-methylsuccinate (**9**) were obtained after hydrolysis of **3** (Fig. 2). The configurations of the products **2a** and **9** were

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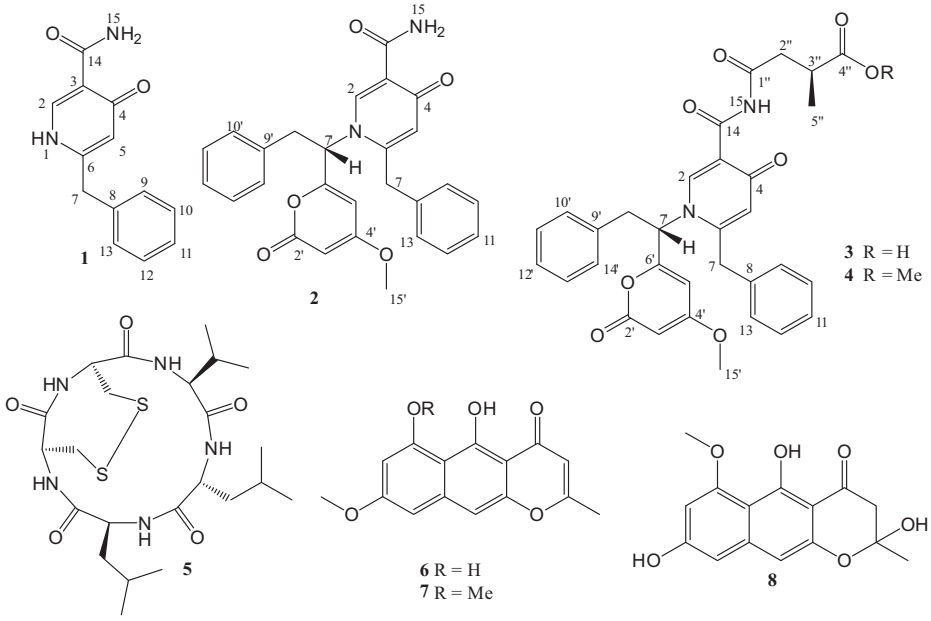

Figure 1. Chemical structures of compounds **1**–**8**.

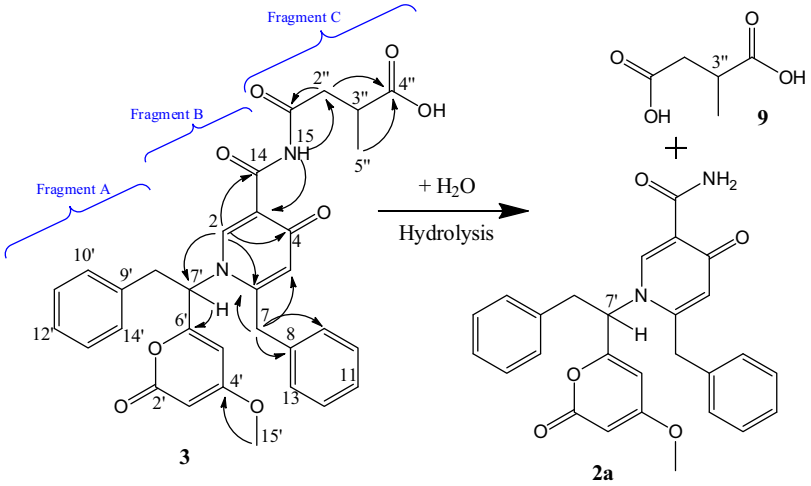

Figure 2. Key HMBC correlations of **3**, and its hydrolysis products.

determined to be 7′*S* and 3″*S*, by comparisons of their OR data with 7′*S*-aspernigrin B (**2**) we obtained and (*S*)-2-methylsuccinate reported in the literature,[11] respectively. So, the configuration of **3** was suggested to be 7′*S*, 3″*S*. The detailed structural elucidation (HR-ESI-MS, NMR and OR analyses) of **3** was showed in Supplementary material.

Aspernigrin D (**4**)[13] was suggested to be the methyl ester of **3**, formed during the isolation or purification processes. The detailed structural elucidation was showed in Supplementary material.

Compound **5** was identified as malformin C with the same absolute configuration reported in the Ref. 14 by its ^{1}H, ^{13}C NMR and OR data ((([α]$_D^{20}$ −24.2, *c* 0.1, $CHCl_3$). Compounds **6**–**8** were identified as rubrofusarin (**6**), rubrofusarin B (**7**), and fonsecin (**8**)[15] by comparison with their ^{1}H and ^{13}C NMR with those reported.

A variety of natural products containing 2-benzyl-4*H*-pyran-4-one and 2-benzylpyridin-4(1*H*)-one substructures have been detected among several Aspergillus stains, and those have been encountered in relatively few fungi outside of the black aspergilli clade.[12] By structurally fragment analysis, it is proposed that the 2-benzylpyridin-4-one-containing metabolites **3** (fragments A + B + C) was derived from pestalamide B (fragments B + C), common product of black aspergilli strains,[12] or **2** (fragments A + B), both of which were biogenetically related to aspernigrin A (**1**, fragment B). Some phenethyl-2-pyrone compounds, such as aspergillusol[16] and pyrophen[14,16] also found in *A. niger*, should be act as important precursor compounds to produce aspernigrins (Fig. 3).

A transmitted/founder virus predominantly uses CCR5 as co-receptor to infect T cells.[17] Isolated compounds were evaluated for their inhibitory effects on infection by CCR5-tropic HIV-1 SF162 in TZM-bl cells (HeLa human cervical carcinoma cells),[18,19] which is a reported cell line, that is, commonly used to measure HIV-1 infection with advantages of being fast and cost-effective.[20] The colorimetric XTT assay was also conducted for the cytotoxicity of these compounds on TZM-bl cells.[21,22] New compound aspernigrin C (**3**) and malformin C (**5**) effectively inhibited infection by HIV-1 SF162 with the effective concentration for 50% inhibition (IC_{50}) being 4.7 ± 0.4 μM and 1.4 ± 0.06 μM, respectively. Malformin C (**5**) showed potent antiviral activity, which was

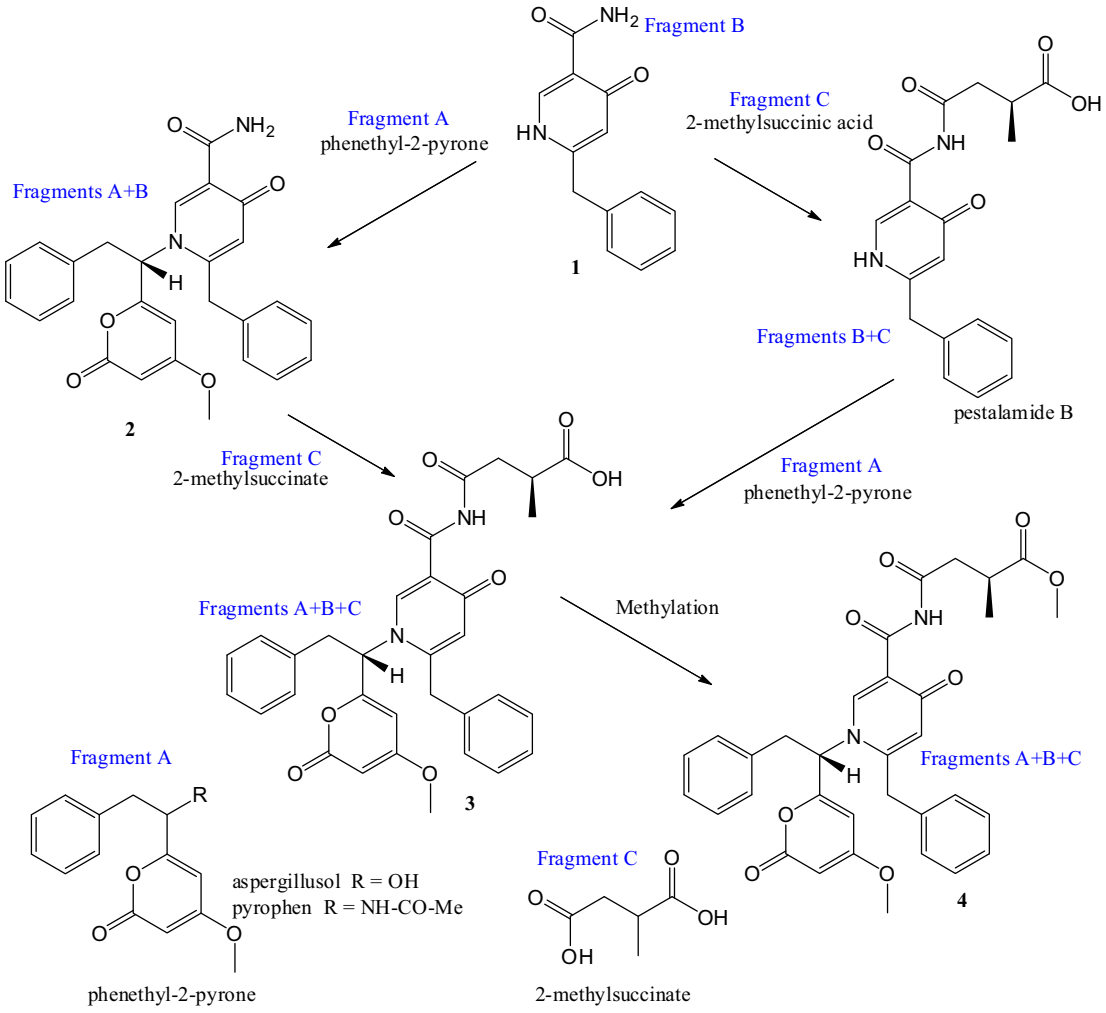

Figure 3. Proposed biogenetical relationships of **1**–**4** and pestalamide B.

comparable to that of abacavir (IC_{50} 0.8 ± 0.1 μM), a nucleoside reverse transcriptase inhibitors, and ADS-J1 (IC_{50} 1.8 ± 0.3 μM), an effective HIV-1 entry inhibitor.[23] While aspernigrin A (**1**) and rubrofusarin (**6**) also exhibited weak antiviral activities with IC_{50} of 83.2 ± 21.4 μM and 56.2 ± 3.3 μM, respectively. All these four compounds showed little or no cytotoxicity to TZM-bl cells at the concentrations tested in the viral infection assay (Table 1 and Fig. 4). Other compounds showed no antiviral activities at the concentration of 50 μM (data not shown).

Structurally unusual 2-benzylpyridin-4-one-containing metabolites, such as aspernigrins A and B, were found to display cytotoxic activities and also strong neuroprotective effect.[6] However, antiviral activities of aspernigrins had never been found before. Now, in our study, the structurally new compound aspernigrin C (**3**) and aspernigrin A (**1**) showed potent and weak efficacy in inhibiting HIV-1 infection, respectively, while aspernigrins B (**2**) and D (**4**) showed no antiviral activities. It is suggested that 2-methylsuccinic acid moiety could remarkably improve the anti-HIV-1 activities of aspernigrins, however, esterification of it resulted in an extremely loss in activity. The completely structure–activity relationship will be determined in the future study.

In addition, a mycotoxin compound malformin C (**5**) displayed potent anti-HIV activity at low μM level, which was comparable to that of abacavir and ADS-J1.[23] Malformin C (**5**) has been found to exert antimicrobial activities, including antibacterial,[24] antimalarial and antitrypanosomal proterties.[25] It is also rediscovered to be promising anti-cancer agents as G2 checkpoint inhibitor.[14] Now, it is the first report of malformin C with its anti-HIV activity.

Table 1
Inhibitory activities of compounds **1**, **3**, **5** and **6** on infection by HIV-1 SF162 and their cytotoxicities to TZM-bl cells[a]

Compounds	IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI[d]
Abacavir	0.8 ± 0.1	ND[e]	ND
ADS-J1	1.8 ± 0.3	ND	ND
Aspernigrin A (**1**)	83.2 ± 21.4	582.6 ± 72.3	7.0
Aspernigrin C (**3**)	4.7 ± 0.4	35.0 ± 2.1	7.5
Malformin C (**5**)	1.4 ± 0.06	16.4 ± 0.3	11.4
Rubrofusarin (**6**)	56.2 ± 3.3	1019.6 ± 199.1	18.1

[a] Two independent experiments were performed in triplicate. Data are presented as mean ± standard deviations.
[b] IC_{50}, the effective concentration for 50% inhibition.
[c] CC_{50}, 50% cytotoxicity concentration.
[d] SI, selectivity index (CC_{50}/IC_{50}).
[e] ND, not done.

In summary, four 2-benzylpyridin-4-one-containing metabolites (**1**–**4**), including two structurally new aspernigrins C (**3**) and D (**4**), were isolated from the marine alga-derived *A. niger* SCSIO Jcsw6F30, together with other four known compounds (**5**–**8**). Aspernigrins C (**3**) and malformin C (**5**) exhibited significant HIV-1 inhibitory activities by SF162 infection in TZM-bl cells. The mechanism of action is on-going and our results suggest these two compounds are potential lead product for the development of anti-HIV therapeutics.

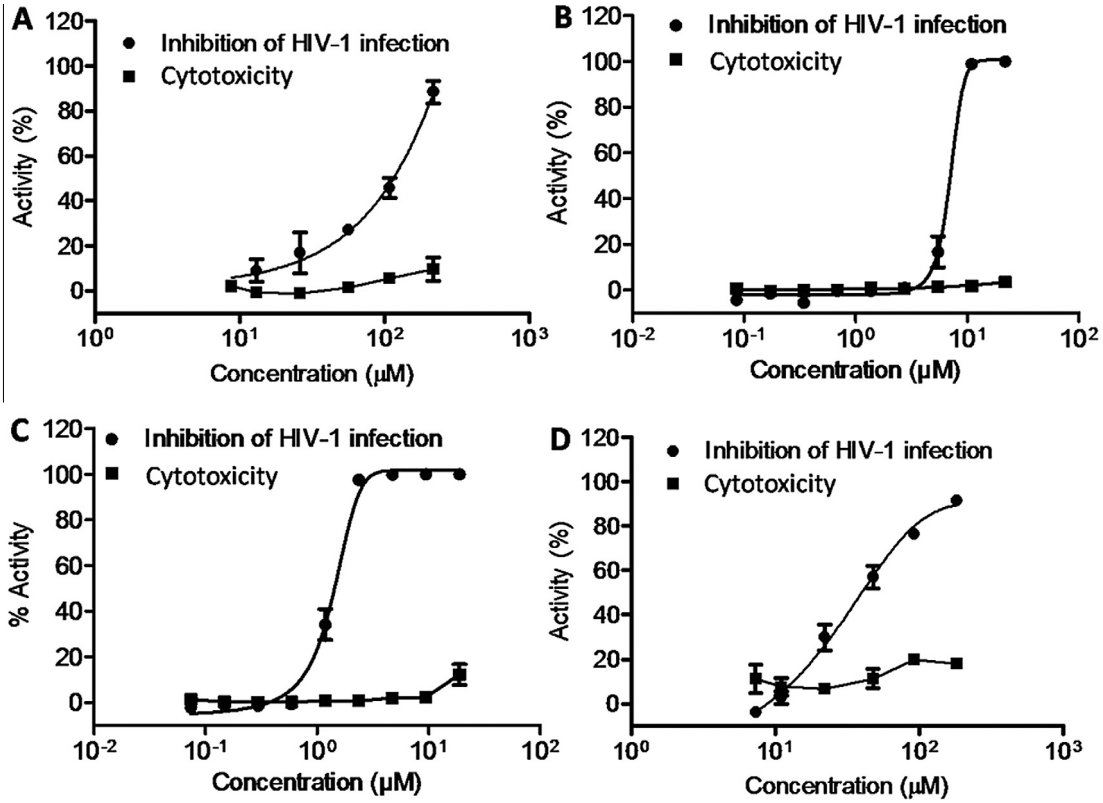

Figure 4. Inhibitory activities of compounds on infection by SF162 and their cytotoxicities to TZM-bl cells. (A) **1**; (B) **3**; (C) **5** and (D) **6**.

Acknowledgments

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Supplementary data

Supplementary data (structural elucidation of compounds **3** and **4**, HR-ESI-MS, 1D and 2D NMR spectral data of compounds **3** and **4**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.005.

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4. Fermentation: Seed medium (consisting of 6.25 g maltose, 6.25 g malt extract, 1 g yeast extract, 6.25 g peptone, 1.25 g potassium dihydrogen phosphate, and 1000 mL distilled water, pH 7.0) in 500 mL Erlenmeyer flasks was inoculated with strain Jcsw6F30 and then incubated at 25 °C for 3 d on a rotating shaker (120 rpm). Production medium (the same as the seed medium) in 500 mL flasks was inoculated with 10% seed solution. The flasks were incubated at 28 °C statically. After 7 d, broth from 100 flasks (15 L) was harvested to isolate substances.
5. Extraction and isolation: The broth (15 L) was extracted with 10 L ethyl acetate stirring three times for 30 min. The ethyl acetate was filtered and then concentrated in vacuo to yield a crude extract (10.5 g). The crude extract was subjected to a silica gel CC and was separated by a linear gradient of petroleum ether (60–90 °C)/EtOAc (50:0, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, and 0:1) to yield eight fractions (fr.I–fr.VIII). Fr.V (petroleum ether/EtOAc 5:1) was dissolved in methanol and chromatographed over semi-preparative HPLC (Sunfire, Prep C_{18} OBD, 10 mm × 250 mm, 5 μm, 7.5 mL/min) with a gradient solvent system from 20% to 60% CH_3CN over 30 min to yield compounds **2** (12 mg, t_R 15.59 min), **3** (6.2 mg, t_R 17.71 min) and **4** (4.6 mg, t_R 20.12 min). Compound **5** (15 mg, t_R 16.73 min) was purified and obtained by same semi-preparative HPLC method in fr.VI. Fr.VII (petroleum ether/EtOAc, 1:1) was purified by silica gel CC ($CHCl_3$/MeOH, gradient elution 100:1–10:1) to afford compounds **1** (8.5 mg), **6** (6.2 mg), **7** (8.9 mg) and **8** (11.5 mg).
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21. Cytotoxicity assay: 100 μL of 5×10^5 cells were cultured overnight and then, 100 μL compound solutions at various concentrations were added. After incubation at 37 °C for 2 days, 50 μL of XTT solution (1 mg/mL) containing 0.02 μM of phenazine methosulfate (PMS) was added. After 4 h, the absorbance at 450 nm was measured with an ELISA reader (Tecan). The CC_{50} values were calculated using the CalcuSyn program.
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