New Anti-inflammatory Cyclopeptides From a Sponge-Derived Fungus Aspergillus violaceofuscus

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INTRODUCTION

As a class of important metabolites from marine-derived organisms, cyclic peptides are extensively present in marine tunicate (Ireland et al., 1982), sponge (Zhang et al., 2010), algae (Xu et al., 2008), bacteria (Teta et al., 2017), fungi (Bao et al., 2013), etc., and often these cyclopeptides possess rare molecular skeleton (Fukuhara et al., 2015). Moreover, due to versatile biological functions including antineoplastic (Ireland et al., 1982), antimicrobial (Teta et al., 2017), anti-inflammatory (Randazzo et al., 2001), antitubercular (Daletos et al., 2015) and histone deacetylase inhibitory activities (Gu et al., 2007), the cyclic peptides have received enduring attention of organic chemists, biologists and pharmacologists. The structures of cyclic peptides may contain unusual amino acids or be modified by methylation (Jang et al., 2017), acetylation, lipidation (Luo et al., 2014), and sulfuration (Fukuhara et al., 2015). These characteristics are playing a vital role in the interactions with relevant bioactive targets (Sieber and Marahiel, 2005; Raaijmakers et al., 2010).

The sponge-derived fungi have been proven to be a prolific source of cyclic peptides (Amagata et al., 2006; Yu et al., 2008). In previous search for structurally unique cyclic peptides from marine sponge-derived fungus Nigrospora oryzae PF18 collected off the Xisha Islands in the South China Sea, we have identified a series of new cyclohexadepsipeptides oryzamides A–C (Ding et al., 2016). As part of our continuing quest for new bioactive molecules, chemical investigation of secondary metabolites of the fungus Aspergillus violaceofuscus from the marine sponge Rentiochalina sp. resulted in the identification of three new cyclopeptides, including a cyclic tetrapeptide violaceotide A (1), an aspochracin-type cyclic tripeptide sclerotiotide L (2), and a new diketopiperazine dimer (3) (Figure 1). Herein, the isolation, structure elucidation and anti-inflammatory studies of the three new cyclic peptides were described.
MATERIALS AND METHODS

General Experimental Procedures
Optical rotations were determined on a Rudolph research analytical autopol VI polarimeter with a 1 dm length cell at room temperature. UV spectra were performed on a Persee TU-1950 UV-VIS spectrophotometer. The NMR spectra were recorded on a Bruker AMX-600 instrument. HRESIMS data were obtained on a Waters Xevo G2-XS Q-Tof mass spectrometer. Reversed-phase HPLC was performed on Waters X-Bridge C18 (5 µm) columns with a Waters 1525 separation module equipped with a Waters 2998 photodiode array detector. MPLC was accomplished using a Interchim PuriFlash 450 chromatography system. Silica gel 60 (200–300 mesh; Yantai, China), Sephadex LH-20 (18–110 µm, Pharmacia Co.) and ODS (50 µm, YMC Co.) were used for column chromatography.

Fungal Strain and Fermentation
The fungus Aspergillus violaceofuscus was isolated from the inner part of the marine sponge Reniochalina sp. collected from the Xisha Islands in the South China Sea. The sample was deposited at the Research Center for Marine Drugs, School of Medicine, Shanghai Jiao Tong University. This strain was identified based on the morphology analyze and ITS gene sequencing (GenBank accession No. FJ491681).

The strain was cultivated on potato dextrose agar at 28°C for 7 days. Large scale fermentation was carried out in 50 erlenmeyer flasks (2 L) each containing 80 g of rice and 120 mL of distilled H2O with 0.3% (m/v) peptone. Each flask was inoculated with 20 mL of cultured broth and incubated under static conditions at room temperature for 40 days.

Extraction and Isolation
The fermented substrate was exhaustively extracted with ethyl acetate to provide the residue (26.0 g) after removal of the organic solvent under reduced pressure.

The EtOAc extract was fractionated by vacuum liquid chromatography on silica gel (200–300 mesh) using CH2Cl2/MeOH gradient elution (500:1–0:1, v/v) to give eleven fractions A–K. Fraction H (6.1 g) was separated by column chromatography (CC) over Sephadex LH-20 eluted with MeOH to afford subfractions H1–H9. Subfraction H3 (1.5 g) was applied to medium pressure liquid chromatography (MPLC) on ODS, eluted with a gradient of 10 to 100% (v/v) MeCN in H2O, to give H3A–H3F. H3D was subjected to reversed-phase HPLC with an elution of 55% MeOH in H2O to give 1 (2.0 mL/min, tR = 23.0 min, 8.6 mg). H3C (31.3 mg) was purified by semi-preparative reversed-phase HPLC eluted with 18% MeCN in H2O to yield 2 (2.0 mL/min, tR = 37.7 min, 2.0 mg). Subfraction H2 (1.82 g) was applied to MPLC on ODS, eluted with a gradient of 10 to 100% (v/v) MeCN in H2O, to give Fr. H2A–H2H. Fraction H2F (169 mg) was then purified by semi-preparative RP-HPLC eluted with 37% MeCN in H2O resulted in the isolation of 3 (2.0 mL/min, tR = 30.5 min, 1.8 mg).

Violaceomide A (1): white amorphous powder; [α]25 D −230 (c 0.6, MeOH); UV (MeOH) λmax (log ε) 220 (3.26), 276 (1.31) nm; HRESIMS m/z 477.2719 [M + H]+ (calcd for C24H37N4O6, 477.2713); 1H and 13C NMR data, Table 1.

Sclerotiotide L (2): pale yellow amorphous powder; [α]25 D −92 (c 0.5, MeOH); UV (MeOH) λmax (log ε) 216 (3.84), 258 (3.98) nm; HRESIMS m/z 481.3042 [M + H]+ (calcd for C24H31N4O6, 481.3026); 1H and 13C NMR data, Table 2.

FIGURE 1 | Structures of compounds 1–3.
Advanced Marfey’s Analysis of Compound 1

Compound 1 (1 mg) were hydrolyzed in HCl (6 M; 1 mL) for 18 h at 110°C. The solutions were then evaporated to dryness and redissolved in H2O (200 µL). The aqueous hydrolysate was added with 1% (w/v) solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (D-FDLA, 100 µL) in acetone and 1 M NaHCO3 (40 µL). After treating at 45°C for 90 min, the reactions were quenched by the addition of HCl (1 M, 40 µL). Appropriate amino acid standards were treated with L-FDLA and D-FDLA as described above and yielded the L-FDLA and D-FDLA standards. Marfey’s derivatives of 1 was subjected to UPLC-MS selected ion chromatograph on a reversed-phase column (Waters ACQUITY HS T3 column; 1.8 µm, 2.1 × 100 mm) with a linear gradient from 10 to 60% aqueous CH3CN containing 0.1% formic acids over 18 min and their retention times were compared with those from the authentic standard derivatives.

Advanced Marfey’s Analysis of Compound 2

Compound 2 (1 mg) were hydrolyzed in HCl (6 M; 1 mL) for 20 h at 110°C. The solutions were then evaporated to dryness and redissolved in H2O (200 µL). The aqueous hydrolysate was divided into two equal portions. One portion was treated with 1% (w/v) solution of 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA, 100 µL) in acetone and 1 M NaHCO3 (40 µL). The second portion was treated with a racemic mixture of a 1% (w/v) solution of 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA, 50 µL) in acetone, 1% (w/v) solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA, 50 µL) in acetone, and 1 M NaHCO3 (40 µL). Both mixtures were heated at 45°C for 90 min and the reactions were quenched by the addition of HCl (1 M, 40 µL). The aliquots were subjected to HPLC-MS selected ion chromatography on a reversed-phase column (Waters XBridge C18 column; 5 µm, 4.6 × 250 mm; 1.0 mL/min) with a linear gradient from 10 to 60% aqueous CH3CN containing 0.1% formic acids over 30 min. The retention times for FDLA derivatives of standard D-Leu and L-Leu were 21.8 and 18.7 min, respectively, while this for FDLA derivatives of compound 3 were 21.8 min.

Anti-inflammatory Assay

THP-1 (a human acute monocytic leukemia cell line) cells (CCTCC) were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and 0.05 mmol/L 2-mercaptoethanol at 37°C in a 5% CO2 and humidified environment. THP-1 cells (5 × 105/mL) were differentiated using 160 nmol/L PMA for 36 h. Differentiation of PMA-treated cells was enhanced by removing the PMA-containing media and the cells were incubated in FBS free, fresh RPMI 1640 for a further 12 h, and then stimulated with compounds or/and LPS at the indicated concentrations and time periods.

Cytokines IL-6, IL-10, MCP-1, and TNF-α in the culture media of THP-1 cells treated with 10 µM compounds or/and 0.1 µg/mL LPS were determined by flow cytometry using the Human Inflammation Cytometric Bead Array (CBA) according to the instruction of the manufacturer (BD Biosciences, San Jose, CA, USA). Cytokine levels were measured on a FACSCalibur flow cytometer (BD Biosciences Pharmingen). The concentrations were assessed by using FCAP Array software.

RESULTS AND DISCUSSION

Compound 1 was isolated as a White, amorphous powder, which possessed a molecular formula of C24H36N6O6 deduced from the pseudomolecular ion peak at m/z 477.2719 [M + H]+ in its HRESIMS (Supplementary Figure 19). The signal distribution pattern observed in the 1H and 13C NMR spectrum (pyridine-d5) (Supplementary Figures 1–2), which showed three exchangeable amide NH signals (δH 9.30, 8.80, 7.20), four amide carbonyls (δC 173.8, 173.6, 173.2, and 171.4), four characteristic α-methylene signals (δH/C 5.14/55.7, 4.74/55.1, 4.47/55.9, and 4.37/65.3) and one N-methyl (δH/C 3.32/30.8), indicated the characteristic of a peptide. Combined analysis of the 2D NMR spectra (Supplementary Figures 3–6) revealed the structures of four amino acid residues, including alanine (Ala), isoleucine (Ile), threonine (Thr), and one tyrosine (Tyr). An HMBC correlation from 4-N-CH3 (δH 3.32) to Ala C-2 (δC 55.1) indicated that the Ala residue was N-methylated. The 24-OCH3 linked to the
### TABLE 1 | $^1$H (600 MHz) and $^{13}$C NMR (150 MHz) Data for 1 in Pyridine-$d_5$.

| Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) | Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) |
|----------|-----------|-----------------|----------|-----------|-----------------|
| N-Me-Ala | 173.6, C  | 65.3, CH         | 12       | 65.3, CH  | 4.37, dd (8.9, 2.4) |
| 1        | 55.1, CH  | 4.78, m          | 14       | 22.2, CH$_3$ | 1.40, d (6.3) |
| 2        | 17.0, CH$_3$ | 1.46, d (7.1)    | 12-NH    | 7.20, brs   |                 |
| 4        | 30.8, CH$_3$ | 3.32, s          | O-Me-Tyr |           |                 |
| Ile      | 173.8, C  | 55.1, CH         | 17       | 35.7, CH$_2$ | 3.81, m; 3.63, m |
| 7        | 25.2, CH$_2$ | 2.00, m; 1.42, m | 11       | 39.7, CH$_2$ | 3.81, m; 3.63, m |
| 9        | 12.4, CH$_3$ | 0.94, t (7.4)    | 19/23    | 131.9, CH | 7.31, d (8.0) |
| 10       |           |                 | 20/22    | 114.9, CH | 7.03, d (8.2) |
| 11       | 173.2, C  |                 | 21       | 159.6, C  |                 |
| 12       |           |                 | 24       | 55.9, CH$_3$ | 3.78, s         |
| Thr      |           |                 | 16-NH    | 9.30, brs |                 |
| Thr      |           |                 | 16-NH    | 9.30, brs |                 |

### TABLE 2 | $^1$H (600 MHz) and $^{13}$C NMR (150 MHz) Data for 2 in CDCl$_3$.

| Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) | Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) |
|----------|-----------|-----------------|----------|-----------|-----------------|
| Ala      | 171.5, C  | 28.6, CH$_2$    | 10-NH    | 6.53, d (7.2) |                 |
| 1        | 55.2, CH  | 4.59, q (7.1)   | 12       | 21.9, CH$_2$ | 1.66, m; 1.57, m |
| 2        | 17.0, CH$_3$ | 1.51, d (7.1)   | 13       | 39.7, CH$_2$ | 3.38, m; 3.06, m |
| N-CH$_3$ | 29.9, CH$_3$ | 3.06, s         | 13-NH    | 6.58, brs   |                 |
| Val      |            | Fatty acid      | 10-NH    | 6.53, d (7.2) |                 |
| 4        | 169.2, C  | 184.8, C        | 11       | 28.6, CH$_2$ | 3.29, m; 1.60, m |
| 5        | 58.8, CH  | 5.11, d (10.5)  | 12       | 21.9, CH$_2$ | 1.66, m; 1.57, m |
| 6        | 27.0, CH  | 2.43, m         | 13       | 39.7, CH$_2$ | 3.38, m; 3.06, m |
| 7        | 20.0, CH$_3$ | 0.92, d (6.3)   | 14       | 137.7, CH | 6.00, d (15.4, 7.8) |
| 8        | 18.0, CH$_3$ | 0.74, d (6.8)   | 15       | 85.5, CH  | 3.62, d (7.8, 3.7) |
| N-CH$_3$ | 30.4, CH$_3$ | 2.95, s         | 16       | 69.5, CH  | 3.90, d (6.5, 3.7) |
| Orn      | 173.1, C  | 18.0, CH$_3$    | 17       | 57.1, CH$_3$ | 3.32, s         |
| 9        |            | 1.12, d (6.5)   | 18       | 24.5, CH  | 1.64, m         |
| 10       | 49.7, CH  | 4.98, t (7.2)   | 19/20    | 57.1, CH$_3$ | 3.32, s         |

### TABLE 3 | $^1$H (600 MHz) and $^{13}$C NMR (150 MHz) Data for 3 in CDCl$_3$.

| Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) | Position | $\delta$C | $\delta$H, mult. ($J$ in Hz) |
|----------|-----------|-----------------|----------|-----------|-----------------|
| 2/2'     | 80.5, CH  | 4.94, s         | 12/12'   | 37.2, CH$_2$ | 3.18, dd (14.0, 9.1) |
| 3/3'     | 59.7, C   | 13/13'          | 13       | 168.5, C  |                 |
| 4/4'     | 130.0, C  | 14/14'          | 15       | 56.3, CH  | 3.77, m         |
| 5/5'     | 124.9, CH | 7.34, d (7.5)   | 16/16'   | 168.2, C  |                 |
| 6/6'     | 119.9, CH | 6.81, t (7.5)   | 17/17'   | 41.8, CH$_2$ | 1.48, m; 2.82, dd (14.0, 8.6) |
| 7/7'     | 129.8, CH | 7.15, t (7.6)   | 18/18'   | 24.5, CH  | 1.64, m         |
| 8/8'     | 110.3, CH | 6.65, d (7.9)   | 19/19'   | 23.1, CH$_3$ | 0.89, d (6.5) |
| 9/9'     | 148.8, C  | 19/19'          | 20/20'   | 21.3, CH$_3$ | 0.87, d (6.5) |
| 11/11'   | 55.8, CH  | 3.99, t (8.8)   | 21       | 57.1, CH$_3$ | 3.32, s         |
benzene ring at C-21 was supported by the HMBC correlation from H$_3$-24 ($\delta_H$ 3.78) to C-21 ($\delta_C$ 159.6). The assignment of the amino acid sequence was carried out by a combination of HMBC, NOESY (Figure 2), and MS/MS analysis. The HMBC correlations from NMeAla H$_3$-4 ($\delta_H$ 3.32) to Ile C-5 ($\delta_C$ 171.4) and from Ile H-6 ($\delta_H$ 5.14) to Thr C-11 ($\delta_C$ 173.2), suggesting a partial sequence of NMeAla-Ile-Thr. The NOESY correlation between OMeTyr NH ($\delta_H$ 9.30) and NMeAla H$_3$-3 ($\delta_H$ 1.46) extended this sequence to OMeTyr-NMeAla-Ile-Thr. The 9º of unsaturation and the molecular formula suggested that 1 was a cyclic peptide. Therefore, the cyclic tetrapeptide ring was closed between OMeTyr and Thr. In addition, the amino acid sequence of 1 was confirmed by mass fragmentation analysis using a quadrupole-time-of-flight (Q-TOF) tandem mass spectrometer (Figure 3). Consequently, the planar structure of 1 was elucidated as a cyclic tetrapeptide with the sequence cyclo-(Thr-O-MeTyr-N-MeAla-Ile).

The absolute configurations of the amino acids were determined by the advanced Marfey’s method after acid hydrolysis (Fujii et al., 1997). Compound 1 was hydrolyzed and then derivatized with L-FDLA. The UPLC-MS comparison between Marfey’s derivatives of the hydrolysate of 1 and appropriate amino acid standards assigned the L configurations for Thr, Tyr, N-Me-Ala, and Ile (Supplementary Figure 23). The final structure of 1 was elucidated as cyclo-[L-Thr–L-O-Me-Tyr–L-N-Me-Ala–L-Ile] and named as violaceotide A.

Compound 2 was isolated as a pale yellow amorphous powder. The molecular formula can be determined as C$_{24}$H$_{40}$N$_4$O$_6$ by HRESIMS ion peak at m/z 481.3042 [M + H]$^+$ (calcld for C$_{24}$H$_{41}$N$_4$O$_6$, 481.3026) (Supplementary Figure 20). The $^1$H NMR spectrum of 2 showed an amide NH proton ($\delta_H$ 6.53), two N-methyl protons ($\delta_H$ 2.95 and 3.06), and three characteristic $\alpha$-methine signals ($\delta_H$ 4.59, 4.98, and 5.11), indicating a tripeptide structure. The $^{13}$C NMR spectrum exhibited a total of 24 carbon resonances, including four amide carbonyl carbons, ten methine carbons, three methene carbons, and seven methyl carbons. The obvious difference in the NMR spectra between compound 2 and sclerotiotide H (Zheng et al., 2010) was the appearance of an additional O-methyl group at $\delta_H$ 3.32 (H-9’) and $\delta_C$ 57.1 (C-9’). The HMBC correlations (Figure 4) from H$_3$-9’ to C-6’ ($\delta_C$
85.5) revealed that the O-methyl was linked to fatty acid chain at position 6′ (Supplementary Figures 7–12). The geometries of the Δ\(^{2,3′}\) and Δ\(^{4,5′}\) olefins were identified as 2′E and 4′E, by the proton spin coupling constants of \(J_{H-2′,H-3′}\) (15.0 Hz) and \(J_{H-4′,H-5′}\) (15.4 Hz). In order to determine the absolute configurations of the amino acid residues of 2, advanced Marfey’s method was utilized (Fujii et al., 1997). HPLC-MS analysis of derivatives of the hydrolysates with D-FDLA and D/L-FDLA method was utilized (Ovenden et al., 2004). The NMR spectra of 2 were elucidated as (2′E,4′E)-cyclo-[(NMe-L-Ala)-(NMe-L-Val)-(N\(_\alpha\)-6′-methoxy-7′-hydroxyocta-2′,4′-dienoyl-L-Orn)] and named as sclerotiotide L.

Compound 3 was isolated as a white amorphous powder. Its molecular formula was determined as C\(_{34}\)H\(_{46}\)N\(_2\)O\(_4\) on the basis of the pseudomolecular ion peak at m/z 597.3177 [M + H\(^+\)] (calcd for C\(_{34}\)H\(_{46}\)N\(_2\)O\(_4\), 597.3189) in the HRESIMS (Supplementary Figure 21), requiring 18 degrees of unsaturation. The NMR spectra of 3 revealed 17 carbon signals, indicating that 3 would be a symmetric homodimer which was further confirmed by its half MS fragment ion peak at m/z 298.2 (Supplementary Figure 22).

In the \(^1\)H NMR spectrum, four aromatic signals at \(\delta_H\) 7.34 (d, \(J = 7.5\) Hz, H-5/H-5′), 7.15 (t, \(J = 7.6\) Hz, H-7/H-7′), 6.81 (t, \(J = 7.5\) Hz, H-6/H-6′), and 6.65 (d, \(J = 7.9\) Hz, H-8/H-8′) and a proton at 4.94 (s, \(\delta_H\) 3.18) to C-15 (\(\delta_C\) 56.3), and C-16 (\(\delta_C\) 168.2) and COSY correlations of H-14 (\(\delta_H\) 5.93)/H-15 (\(\delta_H\) 3.77)/H-17/H-18 (\(\delta_H\) 1.64)/H-19 and H-18/H-20 established a leucine unit (Figure 5). A tryptophan moiety can be concluded from the \(^1\)H NMR signals and the key HMBC correlations from H-12 (\(\delta_H\) 3.18) to C-2 (\(\delta_C\) 80.5), C-3 (\(\delta_C\) 59.7), C-11 (\(\delta_C\) 55.8), and C-13, from H-2 to C-3, and C-9 (\(\delta_C\) 148.8). Ultimately, extensive analysis of the 2D NMR data and comparison of the spectroscopic data with the reported literature (Ovenden et al., 2004) allowed identifying the planar structure of 3 as shown (Supplementary Figures 13–18).

The relative configurations of 3 were deduced from the observed NOESY correlations (Figure 6). The key NOESY correlations of H-11/H-17 and H-11′/H-2′ indicated that these protons were on the same face. The NOESY correlations of H-5′ with H-11 and H-11′ with H-2′ suggested cis-fused ring junction at C-2 and C-3. Marfey’s method (Cho et al., 2018) was employed to determine the absolute configuration at C-15. Compound 3 was hydrolyzed and derivatized with L-FDLA and analyzed by LC-MS to the establishment of absolute configuration of Leu residues.

### Table 4

| Compound | IL-6 (%) | IL-10 (%) | MCP-1 (%) | TNF-α (%) |
|----------|----------|-----------|-----------|-----------|
| 1        | 45.9     | 84.3      | 32.9      | 64.2      |
| 2        | 28.0     | 23.6      | 40.5      | 61.5      |
| 3        | 51.2     | 78.1      | 40.0      | 63.1      |

TABLE 4 | The inhibitory rates of compounds 1-3 against the cytokines expression of LPS-induced THP-1 cells at concentration of 10 \(\mu\)M.
By comparing the retention times of authentic standards of L- and D- forms of Leu, the hydrolysate was identified to contain a unit of D-Leu (Supplementary Figure 25). Therefore, the absolute configurations of 3 were assigned as 2R, 3R, 11S, 15R (Figure 1).

To the best of our knowledge, compound 3 has the same planar structure as an unnamed and ambiguous compound without any spectroscopic data or optical rotation published on a patent (Masashi et al., 1995). Our compound possesses the different stereochemistry with it. Therefore, 3 was reported as a new diketopiperazine dimer herein.

Compounds 1–3 were evaluated their inhibitory activities against the production of four cytokines levels in the serum of human acute monocytic leukemia cell line THP-1 by using the human inflammation cytokometric bead array (CBA) assay (Table 4). The cytokines, including IL-6, IL-10, MCP-1, TNF-α in this study, are pivotal mediators that contribute to inflammation and various related diseases (Li et al., 2017; Wu et al., 2017). Notably, treatment of THP-1 cells by LPS showed a significant elevation in the secretion of the cytokines (P < 0.01). Results showed that the THP-1 cells pretreated with compounds 1 and 3 showed a significant decrease in the LPS-induced expression of IL-10 with inhibitory rates of 84.3 and 78.1% (P < 0.01), respectively. These compounds did not show cytotoxicity against THP-1 cells after 24 h treatment.

CONCLUSIONS

From the marine sponge-derived fungus Aspergillus violaceofuscus, three new cyclic peptides were obtained.

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AUTHOR CONTRIBUTIONS

JL, BG, and LY: performed the experiments; JL: identified the structures and analyzed the data; HL and FY: conceived and designed the experiments; Jl and FY: wrote the paper. All authors listed have approved the work for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00226/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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