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Manadosterols A and B, Sulfonated Sterol Dimers Inhibiting the Ubc13−Uev1A Interaction, Isolated from the Marine Sponge *Lissodendryx fibrosa*

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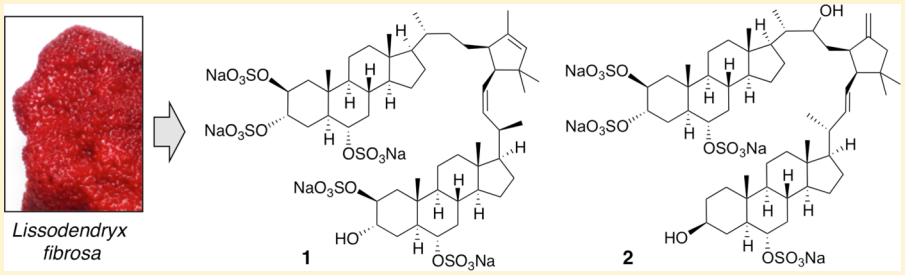
Supporting Information



ABSTRACT: Two new dimeric sterols, manadosterols A (**1**) and B (**2**), were isolated from the marine sponge *Lissodendryx fibrosa* collected in Indonesia. The two compounds are comprised of two sulfonated sterol cores connected through the respective side chains. Manadosterols A (**1**) and B (**2**) inhibited the Ubc13-Uev1A interaction with IC_{50} values of 0.09 and 0.13 μM, respectively. They are the second and third natural compounds showing inhibitory activities against the Ubc13−Uev1A interaction and are more potent than leucettamol A (IC_{50}, 106 μM), the first such inhibitor, isolated from another marine sponge.

The ubiquitin−proteasome pathway consists of the ubiquitin system and the 26S proteasome, a protein degradation machine.[1−4] The ubiquitin system comprises the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) and catalyzes the ubiquitination of client proteins. The successful use of a proteasome inhibitor, Velcade (bortezomib),[5] in anticancer therapy has led to the development of new drugs targeting the ubiquitin−proteasome pathway. In addition to inhibitors targeting the proteasome, much effort has been made to develop inhibitors of E1, E2, and E3 enzymes, and the search for such inhibitors from natural sources has only just begun.[6] Ubiquitination performs both proteolytic and nonproteolytic functions. The lysine48 (K48)-linked polyubiquitin chain is related to proteasome-dependent protein degradation, while the K63-linked chain plays nonproteolytic roles in signal transduction and DNA repair. The formation of the latter chain is catalyzed by a heterodimer formed by the ubiquitin E2 enzyme Ubc13 and either Uev1A or MMS2,[7] and a functional difference between the two Ubc13 complexes was suggested.[8] Thus, Ubc13 exerts E2 activity only after it binds to either Uev1A or MMS2. Recently, it has been reported that Ubc13 is involved in the ubiquitination of p53 bound to polysomes, resulting in a block of p53 tetramerization and subsequent decrease in p53 transcriptional activity, and that the knockdown of Ubc13 led to an increase in p53 activity.[9,10] These reports led us to speculate that an inhibitor of Ubc13, i.e., one preventing the formation of the Ubc13−Uev1A complex, would be a lead for an anticancer agent. We therefore started screening extracts of marine organisms showing inhibitory activity against the formation of the Ubc13−Uev1A complex and, for the first time, succeeded in isolating a natural inhibitor, leucettamol A, from the marine sponge *Leucetta* aff. *microrhaphis*.[11,12] As leucettamol A shows only weak inhibitory activity (IC_{50}, 106 μM (50 μg/mL)), we continued to search

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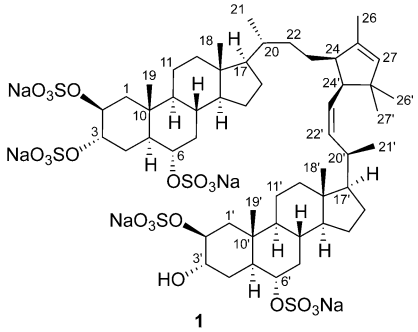
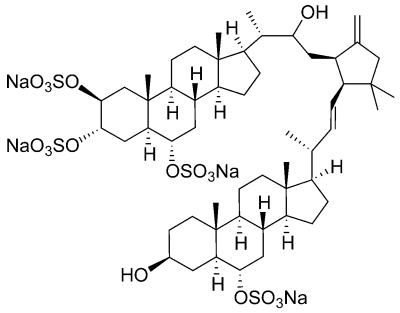
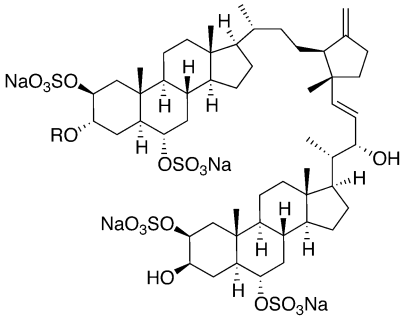
for more potent inhibitors and encountered an active extract of the marine sponge *Lissodendryx fibrosa* collected in Indonesia. Here, we report the isolation and structure determination of two new dimeric sterols, manadosterols A (**1**) and B (**2**), along with inhibitory activities against the formation of the Ubc13−Uev1A complex.



1



2



3: R=H
4: R=SO_3Na

Specimens of *L. fibrosa* were collected in Indonesia. The EtOH extract (7.3 g) of the sponge was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The residual H_2O and *n*-BuOH fractions, which showed inhibitory activity against the formation of the Ubc13−Uev1A complex, were subjected to ODS column chromatography and ODS HPLC to afford manadosterols A (**1**) (1.4 mg, 0.0047%, wet weight) and B (**2**) (7.2 mg, 0.024%), respectively.

ESIMS of manadosterol A (**1**) showed a quasi molecular ion peak at m/z 1319 $[M - Na]^-$, and the molecular formula was determined as $C_{54}H_{83}Na_5O_{21}S_5$ based on HRESIMS. The ^1H NMR spectrum in DMSO-d_6 (Table 1) revealed nine methyl signals [δ 0.61 (s), 0.64 (s), 0.74 (s), 0.84 (s), 0.85 (d, J = 6.6 Hz), 0.87 (s), 0.96 (d, J = 6.6 Hz), 0.97 (s), and 1.58 (s)], six oxymethine signals [δ 3.79 (br s), 3.80 (m), 3.81 (m), 4.08 (br s), 4.35 (br s), and 4.44 (br s)], and three olefin signals [δ 5.15 (br s), 5.13 (dd, J = 10.5, 9.7 Hz), and 5.24 (dd, J = 10.5, 9.4 Hz)]. A dimeric steroidal form connected through the side chains and the presence of five sulfate groups in **1** were readily implied from ^{13}C NMR, COSY, HSQC, and HMBC data and also supported by the molecular formula (Table 1, Figure 1A). HMBC cross-peaks, δ_H 1.58 (H-26)/δ_C 53.2 (C-24), 135.9 (C-27), and 138.1 (C-25), δ_H 5.15 (H-27)/δ_C 46.0 (C-25′) and 138.1 (C-25), δ_H 0.74 (H-26′)/δ_C 28.9 (C-27′), 46.0 (C-25′), 52.9 (C-24′), and 135.9 (C-27), and δ_H 0.97 (H-27′)/δ_C 23.5 (C-26′), 46.0 (C-25′), 52.9 (C-24′), and 135.9 (C-27), completed the planar structure of a cyclopentene ring that was composed of two steroidal side chains. Oxygenation at C-2, C-3, C-6, C-2′, C-3′, and C-6′ was revealed by the interpretation of 2D NMR data, and five sulfate groups could be accommodated at C-2, C-3, C-6, C-2′, and C-6′, as judged by the low-field resonances of C-2 (δ 72.3), C-3 (δ 72.3), C-6 (δ 74.1), C-2′ (δ 74.6), and C-6′ (δ 73.9) versus the resonance of C-3′ (δ 66.2).[13] A 22′*Z*-geometry was established by the coupling constant, $J_{22',23'}$ = 10.5 Hz. NOE correlations assigned all *trans* junctions in the ABCD and A′B′C′D′ rings of **1** and the 20*S*,20′*S*-configurations (Figure 1B). The absolute configurations of the two steroidal skeletons in **1** are assumed to be those of conventional steroids from a biogenetic point of view. The narrow multiplicities for H-2 (δ 4.44, br s), H-3 (δ 4.35, br s), H-2′ (δ 4.08, br s), and H-3′ (δ 3.79, br s) and the lack of NOE correlations, H-2/H_3-19, H-3/H-5, H-2′/H_3-19′, and H-3′/H-5′, showed that H-2, H-3, H-2′, and H-3′ were equatorial. NOE correlations, H_3-27′/H-24 and H-24′, suggested that H-24, H-24′, and H_3-27′ were on the same side of the ring system. On the basis of these data, the structure of **1** was assigned as shown, although the configurations at C-24 and C-24′ relative to the two steroidal cores are arbitrarily drawn.

Manadosterol B (**2**) has the molecular formula $C_{54}H_{84}Na_4O_{18}S_4$ as established by HRESIMS, indicating one less sulfate unit in **2** compared to **1**. Analysis of 2D NMR data (Table S1, Figure 2A) showed that the ABCD rings of **2** have the same structure as those of **1**. The resonance of C-3′ (δ 69.6) revealed a hydroxy group attached to C-3′, and the low-field resonance of C-6′ (δ 73.9) suggested the presence of a sulfate group at C-6′. NOE correlations from H-3′ (δ 3.30) to H-1′ (δ 1.03) and H-5′ (δ 0.87) clearly implied that H-3′ in ring A′ was axial (Figure S11). H-6′ (δ 3.78) showed NOE correlations with H_3-19 and H-8′, which indicated that H-6′ was axial. COSY and HMBC correlations (H-20, H_3-21, and H_2-23/C-22) showed that an additional hydroxy group (δ_H 3.34/δ_C 70.7) was located at C-22. An attempt to determine the absolute configuration of C-22 by the modified Mosher's method was unsuccessful, because the MTPA ester could not be prepared. The 22′*E*-geometry was assigned by the coupling constant, $J_{22',23'}$ = 15.3 Hz. A comparison of the NMR data of **1** and **2** clearly showed that the allylic methyl group (δ_H 1.58/δ_C 15.8) at C-26 of **1** was replaced with a terminal olefin group (δ_H 4.72 and 4.76/δ_C 104.3) in **2**, and NOE correlations indicated that the cyclopentane ring substituents had a *cis* configuration (Figure 2). However, the configuration of the ring could not be determined relative to the two steroidal cores.

Several sulfonated steroidal dimers, fused through their side chains, have been isolated from marine sponges, including crellastatins (from a *Crella* sp.),[13] hamigerols (*Hamigera hamigera*),[14] fibrosterol sulfates A−C (*Lissodendoryx* (*Acanthodoryx*) *fibrosa*),[15] amaroxocanes (*Phorbas amaranthus*),[16] and shishicrellastatins (*Crella* (*Yvesia*) *spinulata*).[17] Among them, fibrosterol sulfates A (**3**) and B (**4**) contain a substituted

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Table 1. NMR Data (500 MHz for ^{1}H and 125 MHz for ^{13}C, DMSO-d_6) for Manadosterol A (1)

no.	δ_C, mult.	δ_H, mult. (J in Hz)	HMBC	no.	δ_C, mult.	δ_H, mult. (J in Hz)	HMBC
1	39.2, CH_2	1.13, m		1′	37.3, CH_2	1.15, m	
		1.74, m				1.88, m	
2	72.3, CH	4.44, br s		2′	74.6, CH	4.08, br s	
3	72.3, CH	4.35, br s	2	3′	66.2, CH	3.79, br s	
4	23.9, CH_2	1.37, m		4′	27.9, CH_2	1.44, m	
		1.90, m	2, 3			1.77, m	2′, 3′
5	43.1, CH	1.40, m	6	5′	43.8, CH	1.33, m	6′
6	74.1, CH	3.80, m		6′	73.9, CH	3.81, m	
7	39.2, CH_2	0.73, m	6	7′	39.2, CH_2	0.76, m	6′
		2.14, m				2.10, m	
8	33.5, CH	1.31, m	6	8′	33.5, CH	1.32, m	6′
9	54.1, CH	0.57, m		9′	54.3, CH	0.58, m	
10	38.0, C			10′	36.0, C		
11	20.4, CH_2	1.20, m		11′	20.4, CH_2	1.14, m	
		1.42, m				1.40, m	
12	39.1, CH_2	1.04, m		12′	38.9, CH_2	1.04, m	
		1.92, m				1.92, m	
13	42.1, C			13′	42.0, C		
14	55.6, CH	1.08, m		14′	55.7, CH	1.15, m	
15	23.8, CH_2	0.98, m		15′	23.8, CH_2	0.98, m	
		1.38, m				1.41, m	
16	27.9, CH_2	1.24, m		16′	27.9, CH_2	1.24, m	
		1.77, m				1.77, m	
17	55.9, CH	1.03, m		17′	56.0, CH	0.99, m	
18	11.8, CH_3	0.61, s	12, 13, 14, 17	18′	11.2, CH_3	0.64, s	12′, 13′, 14′, 17′
19	15.0, CH_3	0.84, s	1, 5, 9, 10	19′	15.0, CH_3	0.87, s	1′, 5′, 9′, 10′
20	35.9, CH	1.30, m		20′	34.0, CH	2.36, m	
21	18.3, CH_3	0.85, d (6.6)	17, 20, 22	21′	21.5, CH_3	0.96, d (6.6)	17′, 20′, 22′
22	32.6, CH_2	1.16, m		22′	138.0, CH	5.24, dd	20′, 24′
		1.41, m				(10.5, 9.4)	
23	29.9, CH_2	0.96, m		23′	127.8, CH	5.13, dd	20′, 24′
		1.23, m				(10.5, 9.7)	
24	53.2, CH	2.27, m		24′	52.9, CH	2.38, m	24, 25, 23′, 25′, 26′, 27′
25	138.1, C			25′	46.0, C		
26	15.0, CH_3	1.58, s	24, 25, 27	26′	23.5, CH_3	0.74, s	27, 24′, 25′, 27′
27	135.9, CH	5.15, br s	25, 25′	27′	28.9, CH_3	0.97, s	27, 24′, 25′, 26′
				3′-OH		4.47, d (3.6)	2′

cyclopentane ring in the connecting side chain similar to manadosterols A (**1**) and B (**2**).

The inhibitory effect on Ubc13−Uev1A interactions was tested by ELISA using purified recombinant Ubc13 and FLAG-Uev1A proteins and a primary anti-FLAG antibody. Manadosterols A (**1**) and B (**2**) inhibited the interaction with IC_{50} values of 0.09 and 0.13 μM (0.12 and 0.16 μg/mL), respectively. The first natural inhibitor of the Ubc13−Uev1A interaction was leucettamol A, a dimeric sphingolipid, isolated by us from the sponge *Leucetta* aff. *microrhaphis*,[11] making manadosterols A (**1**) and B (**2**) the second and third inhibitors isolated from natural sources. It should be noted that **1** is approximately 1000 times more potent than leucettamol A. The recent approval of Velcade (bortezomib), a synthetic proteasome inhibitor, for the treatment of relapsed multiple myeloma has opened the way to the discovery of drugs targeting the ubiquitin−proteasome pathway, consisting of the ubiquitin system, the proteasome, a delivery system for ubiquitinated client proteins, and deubiquitinating enzymes. Although a second generation of proteasome inhibitors and several inhibitors of E1 and E3 enzymes are currently in clinical development,[18,19] further development of inhibitors targeting various components of the ubiquitin−proteasome pathway, including E2 enzymes, by searching natural sources is urgently needed to develop potent and efficient anticancer drugs.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter in MeOH. UV spectra were measured on a JASCO V-550 spectrophotometer in MeOH. IR spectra were measured on a JEOL JIR-6500W spectrophotometer. NMR spectra were recorded on a Bruker Avance 500 or Bruker Avance 600 NMR spectrometer in DMSO-d_6. Chemical shifts were referenced to the residual solvent peaks (δ_H 2.49 and δ_C 39.5) as internal standards. Mass spectra were measured on a Bruker Esquire 3000plus-K1 or Bruker BioTOF-Q mass spectrometer. HPLC was carried out on a Waters 515 HPLC pump and a Waters 2489 UV/visible detector.

Animal Material. The marine sponge was collected at a depth of 5 m in North Sulawesi, Indonesia, in December 2006 and soaked in EtOH immediately. The sponge was identified as *Lissodendryx fibrosa*. A voucher specimen (RMNH POR. 3988) has been deposited in the National Museum of Natural History, The Netherlands.

Extraction and Isolation. The sponge (300 g, wet weight) was extracted with EtOH. The extract (7.3 g) was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The *n*-

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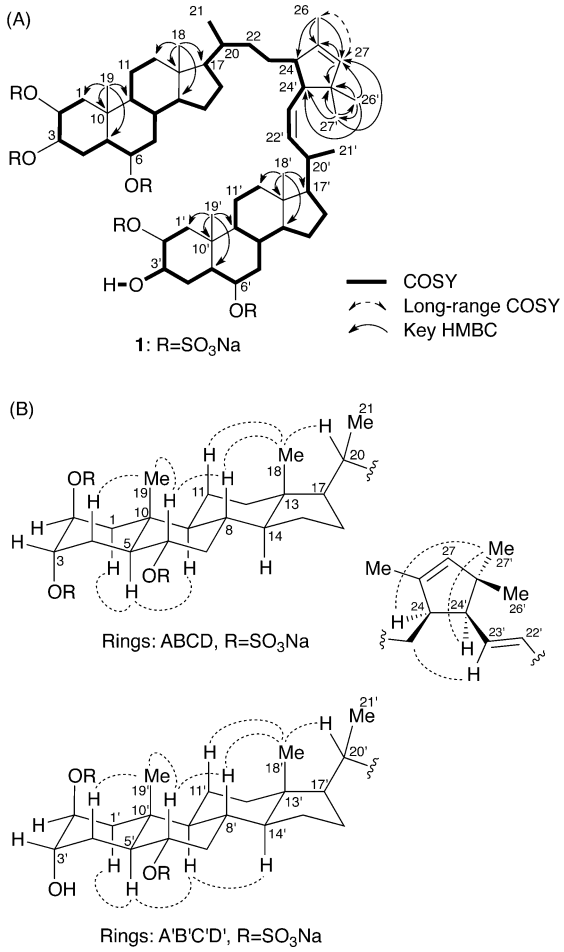

Figure 1. (A) COSY and key HMBC correlations and (B) NOE correlations for **1**.

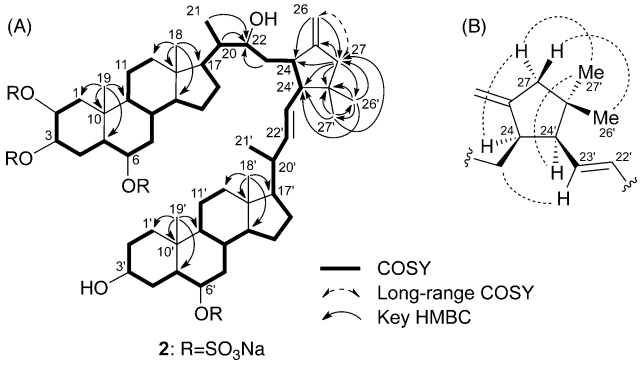

Figure 2. (A) COSY and key HMBC correlations and (B) NOE correlations for the cyclopentene ring of **2**.

BuOH fraction (7.0 g) and H_2O fractions showed inhibitory activity against the formation of the Ubc13−Uev1A complex. The H_2O fraction was subjected to ODS column chromatography with a stepwise gradient using H_2O and MeOH. The fraction that eluted with 70% MeOH−H_2O was purified by ODS HPLC with CH_3CN−H_2O−1 M $NaClO_4$ (4:5:1) and then by Phenyl-Hexyl HPLC (Phenomenex, Luna 5 μm Phenyl-Hexyl column, 250 × 21.2 mm) with CH_3CN−H_2O−1 M $NaClO_4$ (4:5:1) to afford manadosterol A (**1**, 1.4 mg, 0.0047% wet weight). The *n*-BuOH fraction was subjected to ODS column chromatography with a stepwise gradient using H_2O and MeOH. The fraction eluted with 70% MeOH−H_2O was purified by Sephadex LH-20 with 50% MeOH−H_2O followed by ODS HPLC with CH_3CN−H_2O−1 M $NaClO_4$ (4:5:1) to afford manadosterol B (**2**, 7.2 mg, 0.024%).

*Manadosterol A (**1**):* $[\alpha]^{21}_D$ +5.7 (*c* 1.10, MeOH); IR (film) ν_{max} 3462, 2933, 2858, 1602, 1228, 953, 669 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; negative ESIMS *m*/*z* 1319 $[M - Na]^-$, 648 $[M - 2Na]^{2-}$, 424 $[M - 3Na]^{3-}$; HRESIMS *m*/*z* 648.1899 $[M - 2Na]^{2-}$ (calcd for $C_{54}H_{83}Na_3O_{21}S_5$, 648.1862).

*Manadosterol B (**2**):* $[\alpha]^{21}_D$ +24.6 (*c* 0.91, MeOH); IR (film) ν_{max} 3458, 2935, 2862, 1228, 1064, 953, 669 cm^{-1}; ^1H and ^{13}C NMR data, see Table S1; negative ESIMS *m*/*z* 1217 $[M - Na]^-$, 597 $[M - 2Na]^{2-}$, 390 $[M - 3Na]^{3-}$; HRESIMS *m*/*z* 597.2125 $[M - 2Na]^{2-}$ (calcd for $C_{54}H_{84}Na_2O_{18}S_4$, 597.2168).

Ubc13−Uev1A Interaction Inhibition Assay. *Escherichia coli* BL21 cells transformed with pGEX6P1-Ubc13 or pGEX6P1-FLAG-Uev1A were precultured overnight at 37 °C in LB medium supplemented with 100 μg/mL ampicillin, transferred to a 20-fold volume of the same medium, and cultured at 37 °C for 1.5 h.[11,20] Isopropyl 1-thio-β-D-galactoside was then added at a final concentration of 0.1 mM, and the cells were further cultured at 25 °C for 6 h. Two GST-fused proteins were purified by using glutathione-immobilized agarose beads, and the GST tag of GST-Ubc13 or GST-FLAG-Uev1A was removed by cleavage with PreScission protease (GE Healthcare). The inhibition of the Ubc13−Uev1A interaction was tested by ELISA with a 96-well plate (F96 maxisorp immuno plate) (Nunc). Human Ubc13 diluted in phosphate-buffered saline (PBS) was coated onto a 96-well plate and incubated at 4 °C overnight. The wells were extensively washed with 0.05% Tween 20 in PBS (PBST) and incubated with 5% bovine serum albumin (BSA) (Sigma) in PBS at 37 °C for 1.5 h. After washing with PBST, the wells were incubated for 1.5 h with a mixture of FLAG-Uev1A and a test sample diluted in PBS that had been previously incubated at 37 °C for 15 min. The wells were thoroughly washed with PBST and incubated with anti-FLAG M2 monoclonal antibody (Sigma) in 5% BSA in PBST for 1.5 h, followed by the second antibody (mouse IgG-HRP) (Amersham) in 5% BSA in PBST for 1.5 h. After washing with PBST and then citrate−phosphate buffer (pH 5.0), a mixture of *o*-phenylene diamine and 0.007% H_2O_2 in the citrate-phosphate buffer was added to the wells, and the wells were incubated at 37 °C for 30 min. Finally, 2 M H_2SO_4 was added to the wells and the optical density at 490 nm was measured on a microplate reader. The IC_{50} value, the concentration required for 50% inhibition of the Ubc13−Uev1A interaction, was calculated from the results of duplicate experiments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR spectra of **1** and **2**. These materials are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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