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Highly Oxygenated Meroterpenoids from Fruiting Bodies of the Mushroom *Tricholoma terreum*

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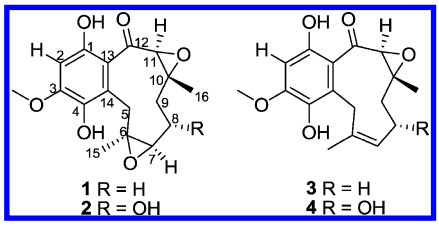
Supporting Information

ABSTRACT: Four new meroterpenoids, terreumols A–D (**1**–**4**), with a rare 10-membered ring system, were isolated from the fruiting bodies of *Tricholoma terreum*. Their structures with absolute stereochemistry were determined by comprehensive spectroscopic methods, as well as single-crystal X-ray diffractions. Compounds **1**, **3**, and **4** were evaluated for their cytotoxicities against five human cancer cell lines; all of them exhibited inhibitory effects, with IC_{50} values comparable to those of cisplatin.

Meroterpenoids are broadly defined as compounds of mixed biosynthetic origin (polyketide-terpenoid) containing either a phenolic, a quinine, or a closely related subunit linked to a terpenoid moiety by at least one C–C bond,[1] which have been found in plants, marine organisms, and higher fungi. More significantly, the family has the reputation for displaying potent biological activities, and members have been reported to be anti-inflammatory,[2] antimalarial,[2a] antioxidants,[2b,3] antitumor,[2d,4] antibacterials,[1e,4c,5] and *ras* farnesyl-protein transferase inhibitors[6] and to show severe ichthyotoxic effects.[7] Besides their interesting pharmacological properties, they have complicated biosynthetic origins and arouse interest from synthetic chemists.[1e,8]

The wild mushroom *Tricholoma equestre* (or *T. flavovirens*) has caused poisoning in southwestern France. Twelve people were hospitalized for severe weakness and muscle loss after eating the mushrooms, and three of them died.[9] Despite the highly toxic nature of *T. equestre*, the poison in this mushroom has not been characterized. This inspired us to investigate the toxic constituents of *T. equestre* and related species. When we collected *T. equestre*, the mushroom *Tricholoma terreum*, another species of the same genus, was harvested from the same environment. Since *T. equestre* could be mistaken for other members of the *Tricholoma* genus or similar myotoxic effects could be caused by other related fungus, *T. terreum* was tested and investigated for the suspected myotoxicity.

In this paper, we report four rare meroterpenoids, terreumols A–D (**1**–**4**), from the basidiomycete *T. terreum*. Notably, terreumols A–D (**1**–**4**) contain two C–C bonds between a benzene ring and the terpenoid moieties, building a rare 10-membered carbon ring that has been previously reported in wigandol from leaves and flowers of *Wigandia kunthii*,[10] flavidulols from the mushroom *Lactarius flavidulus*,[11] and globiferin from *Cordia globifera*.[12] The absolute configuration of terreumols A–D (**1**–**4**) was determined by X-ray diffraction. These compounds showed cytotoxic activity.



RESULTS AND DISCUSSION

Terreumol A (**1**) was isolated as yellow crystals. The molecular formula $C_{17}H_{20}O_6$ was determined by HREIMS at m/z 320.1258 (calcd for $C_{17}H_{20}O_6^+$, 320.1260), corresponding to eight degrees of unsaturation. The IR data at 3440 cm^{-1} revealed the presence of hydroxy groups, while the UV absorptions at 372 and 291 nm suggested the presence of a multiconjugated moiety. The 1D NMR spectra, as well as the HSQC spectrum, revealed 17 carbon resonances, which were ascribed to three methyls (including one methoxy), three methylenes, three methines (including one olefinic carbon), and eight quaternary carbons (Table 1). Overall consideration of the NMR data suggested that compound **1** was a meroterpenoid similar to wigandol,[10] but highly oxygenated. In detail, one olefinic proton at δ_H 6.52 (1H, s, H-2), together with six olefinic carbons at δ_C 159.2 (s, C-1), 99.7 (d, C-2), 155.0 (s, C-3), 139.7 (s, C-4), 115.2 (s, C-13), and 123.6 (s, C-14), indicated the presence of one five-substituted benzene ring. One downfield signal of a hydroxy proton at δ_H 11.97 (1H, s, OH-1) indicated the existence of one intramolecular hydrogen bond. In addition the proton (δ_H 11.97, s, OH-1) showed HMBC correlations to C-1, C-2, C-13 and δ_C 199.6 (s, C-12), indicating the carbonyl carbon (C-12) to be connected with the benzene ring at C-13, which was also supported by the UV absorptions at

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Table 1. ^{1}H NMR Data (600 MHz) of 1−4 (δ in ppm, J in Hz)

	1[a]		2[b]		3[a]		4[b]	
no.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		159.2, qC		159.1,qC		159.4, qC		159.0, qC
2	6.52, s	99.7, CH	6.45, s	99.3, CH	6.50, s	99.4, CH	6.43, s	98.9, CH
3		155.0,qC		153.4, qC		154.9, qC		153.0, qC
4		139.7, qC		138.5, qC		139.5, qC		138.0, qC
5	3.44, d (15.1)	29.8, CH_2	3.60, d (15.8)	29.9, CH_2	3.84, d (15.1)	30.3, CH_2	3.91, d (15.0)	30.8, CH_2
	2.77, d (15.1)		2.59, d (15.8)		3.54, d (15.1)		3.24, d (15.0)	
6		60.4, qC		61.7, qC		136.9, qC		135.5, qC
7	2.85, dd (9.8, 3.8)	66.1, CH	2.80, d (8.0)	70.2, CH	5.43, t (8.3)	125.0, CH	5.28, d (6.6)	130.2, CH
8	2.27, m	25.5, CH_2	3.77, m	69.0, CH	2.51, m	23.2, CH_2	4.73, brt (9.6)	66.1, CH
	1.45, m				2.06, overlap			
9	2.33, dd (12.7, 7.3)	34.9, CH_2	2.47, m	43.4, CH_2	2.26, dd (13.2, 6.6)	38.1, CH_2	2.45, d (12.4)	46.6, CH_2
	1.43, m		1.79, t (13.0)		1.12. t (13.2)		1.49, d (12.4)	
10		64.7, qC		62.0, qC		64.3, qC		62.0, qC
11	3.89, s	64.4, CH	3.79, s	63.6, CH	4.15, s	63.8, CH	4.10, s	63.2, CH
12		199.6, qC		197.9, qC		201.1, qC		199.2, qC,
13		115.2, qC		114.7, qC		115.5, qC		114.7, qC
14		123.6, qC		121.0, qC		124.5, qC		122.4, qC
15	1.33, s	21.5, CH_3	1.36, s	21.4, CH_3	1.77, s	21.5, CH_3	1.75, s	21.3, CH_3
16	1.38, s	16.4, CH_3	1.45, s	17.1, CH_3	1.40, s	16.5, CH_3	1.45, s	17.4, CH_3
1-OH	11.97,s		11.91, s		12.15, s		12.09, s	
4-OH	7.85, s		5.62, s		7.65, s		5.52, s	
-OCH_3	3.96, s	56.6, CH_3	3.95, s	56.5, CH_3	3.96, s	56.6, CH_3	3.94, s	56.4, CH_3

[a]Spectra were measured in acetone-d_6. [b]Spectra were measured in $CDCl_3$.

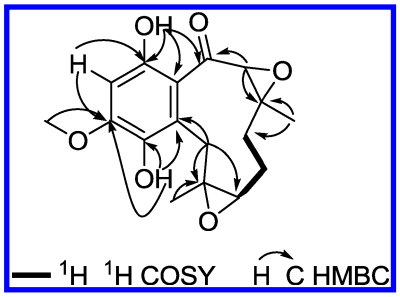

Figure 1. Selected 2D NMR correlations of 1.

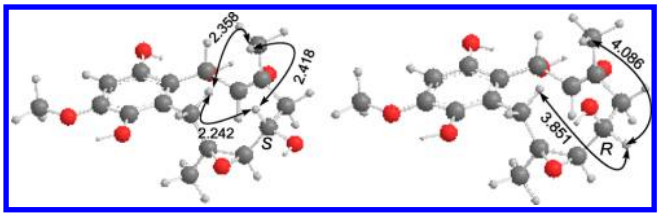

Figure 2. Key ROESY correlations and Chem3D structures showing stereoconfiguration of C-8 in 2.

372 and 291 nm. In the HMBC spectrum (Figure 1), the correlations from another hydroxy proton at δ_H 7.85 (1H, s, OH-4) to δ_C 139.7 (s, C-4), 155.0 (s, C-3), and 123.6 (s, C-14), from δ_H 3.96 (3H, s, OCH_3) to C-3, and from H-2 to C-3 allowed the hydroxy and the methoxy to be assigned at C-4 and C-3, respectively. The remaining 10 carbons (except for the above seven carbons consumed by the benzene and the methoxy group) were readily assigned to a monoterpenoid moiety, showing similarities to that of wigandol,[10] which formed a 10-membered ring system. The four oxygenated carbons at δ_C 60.4 (s, C-6), 66.1 (d, C-7), 64.7 (s, C-10), and 64.4 (d, C-11) suggested that the 1,5-diene in wigandol was replaced with two epoxy moieties in **1**, as well as by the MS data; this was confirmed by analysis of the HMBC and ^{1}H−^{1}H COSY correlations (Figure 1). Detailed analysis of 2D NMR data identified the planar structure of **1**. The ROESY spectrum could not completely determine the stereochemistry of **1** because of the 10-membered ring in the structure. Fortunately, an X-ray diffraction not only confirmed the planar structure of **1** but also determined the absolute configuration as shown in Figure 3.

Terreumol B (**2**) was isolated as a yellow powder. The HREIMS displayed an $[M]^+$ peak at m/z 336.1216 (calcd 336.1209), which analyzed for $C_{17}H_{20}O_7$, 16 mass units more than that of **1**. The UV absorptions at 362 and 286 nm together with the ^{13}C NMR (Table 1) data were quite similar to those of compound **1**, except that the methylene at δ_C 25.5 (C-8) was oxidized into a methine at δ_C 69.0, resulting in a downfield shift of C-7 ($\Delta\delta$ 4.1 ppm) and C-9 ($\Delta\delta$ 8.5 ppm) in compound **2**, consistent with the ^{1}H−^{1}H COSY correlations of H-7/H-8/H-9. Detailed analysis of 1D and 2D NMR data (HSQC, HMBC, ^{1}H−^{1}H COSY) suggested that the remainder of **2** was identical to that of **1**. Biogenetically, the absolute configurations of C-6, C-7, C-10, and C-11 in **2** would be expected to be the same as those of **1**. In the ROESY spectrum, the correlations among H-5_b, H-8, and H_3-16 were observed (Figure 2), which allowed H-8 to be placed on the same side as H_3-16. In addition only the *S* form can satisfy the ROESY correlations mentioned above, as shown by 3D structure models (Figure 2). Therefore, compound **2** was established as terreumol B, as shown.

Terreum C (**3**), isolated as yellow needle crystals from acetone, had the molecular formula $C_{17}H_{20}O_5$, according to its HREIMS at m/z 304.1327 ($[M]^+$, calcd 304.1311), 16 mass units less than that of **1**. The UV absorptions at 371 and 291 nm indicated that the multiconjugated moiety remained. By intensive comparison of the ^{1}H and ^{13}C NMR data (Table 1) with those of **1**, the significant differences were the absence of two oxygenated carbon signals in **3** and the presence of a

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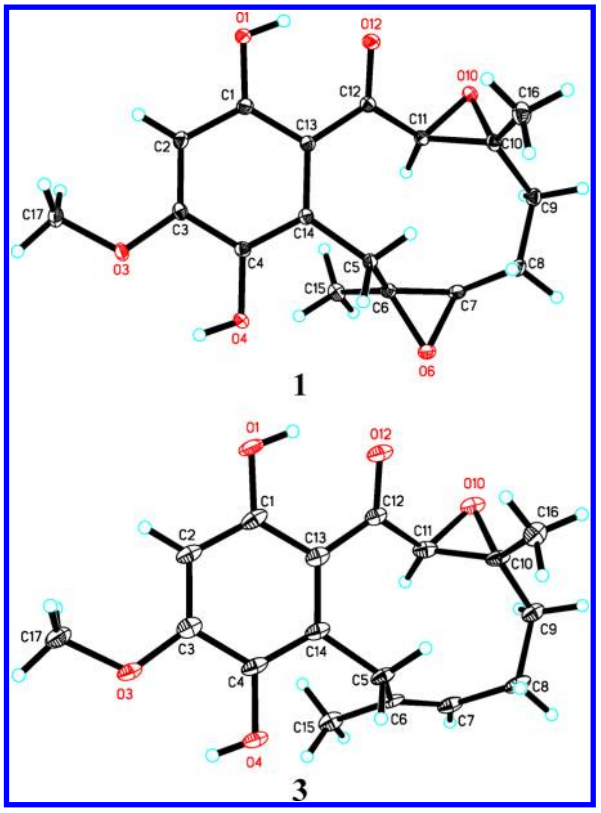

Figure 3. X-ray structures of **1** and **3**.

trisubstituted carbon double bond (δ_C 136.9, s; δ_C 125.0, d, δ_H 5.43, t, J = 8.3 Hz). In the $^1H-^1H$ COSY spectrum, the correlations of H-7/H-8 located the double bond between C-6 and C-7, which was further supported by the HMBC correlations from H-7 (δ_H 5.43, t, J = 8.3 Hz) to C-5 (δ_C 30.2, t), C-8 (δ_C 23.2, t), and C-9 (δ_C 38.1, t). Finally, the X-ray diffraction determined the absolute structure of **3** as shown in Figure 3.

Terreum D (**4**) had an $[M]^+$ peak at m/z 320.1258 ($C_{17}H_{20}O_6$) in the HREIMS, 16 mass units more than that of **3**. Compound **4** was readily identified as the 8-OH derivative of **3** from ^1H and ^{13}C NMR data at δ_C 66.1 (d, C-8) and δ_H 4.73 (1H, t, J = 9.6 Hz, H-8) (Table 1), which was confirmed by the HMBC correlations from H-8 to C-6 (δ_C 135.5, s), C-7 (δ_C 130.2, d), C-9 (δ_C 46.6, t), and C-10 (62.0, s). Detailed analysis of the 2D NMR (HSQC, HMBC, and ROESY) data confirmed that the other parts were the same as those of **3**. Finally, C-8 was determined to be *S* configured, the same as that of **2**. Thus, the structure of compound **4** was elucidated as terreum D, as shown.

Compounds **1**, **3**, and **4** were evaluated for cytotoxicity against five human cancer cell lines using the MTT method as reported previously.[13] All three compounds showed comparable cytotoxicities with those of cisplatin, and their IC_{50} values are presented in Table 2.

Table 2. Cytotoxicities of Compounds 1, 3, and 4 (IC_{50}, μmol)

compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	4.0	11.3	4.2	16.1	14.2
3	5.6	24.7	16.4	24.6	>40
4	17.8	14.9	6.5	21.0	21.1
cisplatin	1.1	6.7	6.0	15.4	16.3

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were obtained on a Yuhua X-4 digital microdisplaying melting point apparatus. Optical rotations were recorded on a JASCO P-1020 digital polarimeter. UV and the IR spectra were obtained on a Shimadzu UV2401PC and a Bruker Tensor 27 FT-IR spectrometer with KBr pellets. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 600 MHz spectrometer with tetramethylsilane (TMS) as an internal standard at room temperature. High-resolution (HR) EIMS were recorded on a Waters AutoSpec Premier P776. X-ray diffractions were performed on an APEX DUO spectrophotometer. Silica gel (200−300 mesh, Qingdao Marine Chemical Ltd., People's Republic of China) and reverse-phase C_{18} (RP-18) gel (20−45 μm, Fuji Silysia Chemical Ltd., Japan) were used for open column chromatography (CC). Preparative HPLC was performed on an Agilent 1100 liquid chromatography system equipped with a Zorbax SB-C_{18} column (9.4 mm × 150 mm). Fractions were monitored by TLC. Spots were visualized by heating silica gel plates immersed in vanillin−H_2SO_4 in ethanol.

Fungi Sample. The mushroom *Tricholoma terreum* was collected from southwestern France in 2011 and identified by Prof. Zhu-Liang Yang, Kunming Institute of Botany, Chinese Academy of Sciences. A specimen (No. kib20111212) was deposited at Kunming Institute of Botany, Chinese Academy of Sciences. The collection was permitted by the local forest authority.

Extraction and Isolation. The dried fruiting bodies of *T. terreum* (1.0 kg) were extracted with $CHCl_3$−MeOH (1:1). The extract was separated by solvent partition between EtOAc and water. The EtOAc layer was concentrated under reduced pressure to give a crude extract (23 g), and this residue was subjected to silica gel CC, eluted with a gradient of $CHCl_3$−MeOH (1:0 → 0:1), to obtain seven fractions (A−G). Fraction C was separated by RP-18 (MeOH−H_2O, 50−100%) to give 14 subfractions (C1−C14). Fraction C6 was subjected to silica gel CC (petroleum ether−EtOAc, 4:1) and further purified by semipreparatiive HPLC (15−40%, MeCN−H_2O, 10 mL/min, 30 min) to yield **2** (0.8 mg) and **4** (1.0 mg). Fraction C7 was purified by silica gel CC (petroleum ether−EtOAc, 6:1) to obtain **1** (5.9 mg). Finally, fraction C8 was purified by semipreparative HPLC (30−50%, MeCN−H_2O, 10 mL/min, 20 min) to afford **3** (1.7 mg).

*Terreumol A (**1**):* yellow crystals (aqueous acetone); mp 216−218 °C; $[\alpha]_D^{20}$ −216.1 (*c* 0.29, MeOH); IR (KBr) ν_{max} 3440, 1622, 1440, 1385, 1256, 1202, 1084, 930 cm^{-1}; UV (MeOH) λ_{max} (log ε) 372 (3.10), 291 (3.24), 249 (3.15), 202 (3.50) nm; ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (Me_2CO-d_6), see Table 1; positive ion HREIMS m/z 320.1258 (calcd for $C_{17}H_{20}O_6$ $[M]^+$, 320.1260).

*Terreumol B (**2**):* yellow oil; $[\alpha]_D^{20}$ −9.3 (*c* 0.08, MeOH); IR (KBr) ν_{max}3440, 2922, 1709, 1625, 1485, 1441, 1204, 942 cm^{-1}; UV (MeOH) λ_{max} (log ε) 363 (2.32), 286 (2.67), 242 (2.66), 204 (2.99) nm; ^1H (600 MHz) and ^{13}C NMR (150 MHz) data ($CDCl_3$), see Table 1; positive ion HREIMS m/z 336.1216 (calcd for $C_{17}H_{20}O_7$ $[M]^+$, 336.1209).

*Terreumol C (**3**):* yellow crystals (aqueous acetone); mp 167−169 °C; $[\alpha]_D^{20}$ −55.5 (*c* 0.17, MeOH); IR (KBr) ν_{max} 3433, 2923, 1695, 1622, 1486, 1440, 1384, 1258, 1204, 944 cm^{-1}; UV (MeOH) λ_{max} (log ε) 371 (2.99), 291 (3.15), 249 (3.11), 204 (3.61) nm; ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (Me_2CO-d_6), see Table 1; positive ion HREIMS m/z 304.1327 (calcd for $C_{17}H_{20}O_5$ $[M]^+$, 304.1311).

*Terreumol D (**4**):* yellow powder; $[\alpha]_D^{20}$ −37.3 (*c* 0.10, MeOH); IR (KBr) ν_{max}3440, 2924, 1623, 1486, 1460, 1385, 1266, 1203, 601 cm^{-1}; UV (MeOH) λ_{max} (log ε) 371 (3.01), 291 (3.19), 249 (3.16), 204 (3.68) nm; ^1H (600 MHz) and ^{13}C NMR (150 MHz) data ($CDCl_3$), see Table 1; positive ion HREIMS m/z 320.1258 (calcd for $C_{17}H_{20}O_6$ $[M]^+$, 320.1260).

X-ray Crystallographic Analysis of 1 and 3. Compound **1** was obtained as yellow crystals from acetone. The crystal structure was solved by direct methods using SHELXS-97 (Sheldrick, 1990) with Cu Kα radiation, expanded using geometrical calculations and difference Fourier techniques, and refined using full-matrix least-squares on F^2. Non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were located by geometry and refined as riding atoms with relative isotropic parameters. CCDC 935079 (**1**) and CCDC 935078 (**3**)

contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/conts/retrieving.html.

Crystal data for **1**: $C_{17}H_{20}O_6$, M = 320.33, orthorhombic, a = 13.1252(4) Å, b = 13.5105(4) Å, c = 17.3945(5) Å, α = 90.00°, β = 90.00°, γ = 90.00°, V = 3084.53(16) Å^3, T = 100(2) K, space group $P2_12_12_1$, Z = 8, μ(Cu Kα) = 0.873 mm^{-1}, 14 776 reflections measured, 5280 independent reflections (R_{int} = 0.0452). The final R_1 values were 0.0352 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0965 ($I > 2\sigma(I)$). The final R_1 values were 0.0353 (all data). The final $wR(F^2)$ values were 0.0967 (all data). The goodness of fit on F^2 was 1.072. Flack parameter = 0.04(11). The Hooft parameter is 0.03(5) for 2139 Bijvoet pairs.

Crystal data for **3**: $C_{17}H_{20}O_5$, M = 304.33, orthorhombic, a = 12.9578(3) Å, b = 13.5937(3) Å, c = 17.1115(4) Å, α = 90.00°, β = 90.00°, γ = 90.00°, V = 3014.10(12) Å^3, T = 100(2) K, space group $P2_12_12_1$, Z = 8, μ(Cu Kα) = 0.813 mm^{-1}, 12 088 reflections measured, 5005 independent reflections (R_{int} = 0.0515). The final R_1 values were 0.0891 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.2688 ($I > 2\sigma(I)$). The final R_1 values were 0.0915 (all data). The final $wR(F^2)$ values were 0.2730 (all data). The goodness of fit on F^2 was 1.267. Flack parameter = 0.0(3). The Hooft parameter is 0.11(11) for 2040 Bijvoet pairs.

Cytotoxicity Assay. Five human cancer cell lines, breast cancer MCF-7, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, colon cancer SW480, and lung cancer A-549 cells, were used in the cytotoxicity assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates.[13] Briefly, 100 μL of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with an initial density of 1×10^5 cells/mL. Each tumor cell line was exposed to the test compound dissolved in DMSO at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μmol in triplicates for 48 h, with cisplatin (Sigma, USA) and vinorelbine (National Institute for the Control of Pharmaceutical and Biological Products, P. R. China) as positive controls. After compound treatment, cell viability was detected and a cell growth curve was graphed. IC_{50} values were calculated by Reed and Muench's method.[14]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of MS and ^{1}H, ^{13}C, and 2D NMR spectra for compounds **1**−**4**. This material is 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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