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Functionally Optimized Neuritogenic Farinosone C Analogs: SAR-Study and Investigations on Their Mode of Action

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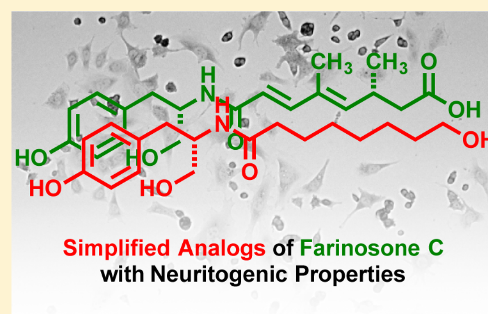
Supporting Information

ABSTRACT: Several natural products derived from entomopathogenic fungi have been shown to initiate neuronal differentiation in the rat pheochromocytoma PC12 cell line. After the successful completion of the total synthesis program, the reduction of structural complexity while retaining biological activity was targeted. In this study, farinosone C served as a lead structure and inspired the preparation of small molecules with reduced complexity, of which several were able to induce neurite outgrowth. This allowed for the elaboration of a detailed structure−activity relationship. Investigations on the mode of action utilizing a computational similarity ensemble approach suggested the involvement of the endocannabinoid system as potential target for our analogs and also led to the discovery of four potent new endocannabinoid transport inhibitors.



KEYWORDS: *Neurite outgrowth, natural products, endocannabinoid membrane transport, CB_1 receptor, SAR, truncated*

Neurodegenerative disorders are among the leading causes of death in aging societies, and every third person dying suffers from a type of dementia. To date, approved medication slows down the progress or reduces the symptoms of dementia.[1] Consequently, neurite-growth-promoting and neuroprotective compounds are considered as molecular approaches for the restoration of brain function.[2,3] Several natural products have been reported to induce neurite outgrowth, such as withanolide A,[4−6] the gentiside family,[7,8] gelsemiol[9,10] or jiadifenolide.[11] Often, the structural complexity of natural products is directly associated with their challenging and time-consuming synthesis.[12−14] Therefore, structural simplification while maintaining or even increasing their respective bioactivity has emerged as an important strategy in natural products research ("reduce to the maximum")[15] and is crucial to facilitate the supply of beneficial small molecules. Wender introduced the concept of function-orientated synthesis, which is based on the reduction of molecular complexity by retained function (bioactivity), resulting in increased step economy.[16] Truncation of complex natural products as exemplified by the modification of halichondrin B resulted in a drug in clinical use.[17,18] In the area of neuritogenic natural products, we[19] and others[20] have recently provided functionally optimized scaffolds for neuronal differentiation.

Our group accomplished the total synthesis of several neuritogenic alkaloids originating from entomopathogenic fungi such as pretenellin (**1**), farinosone A (**2**), or farinosone C (**3**) and assigned their absolute configuration (Figure 1).[9,10,21−23] While the biosynthesis of these compounds is likely to be related,[24,25] their synthesis required at least 14 steps.[22] From a

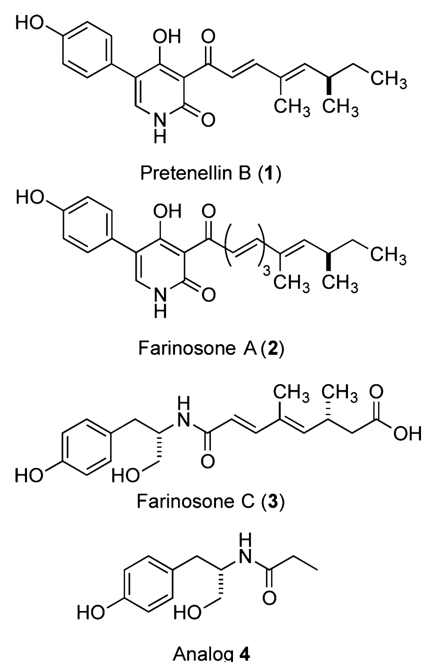


Figure 1. Neuritogenic natural products and first analog **4**.

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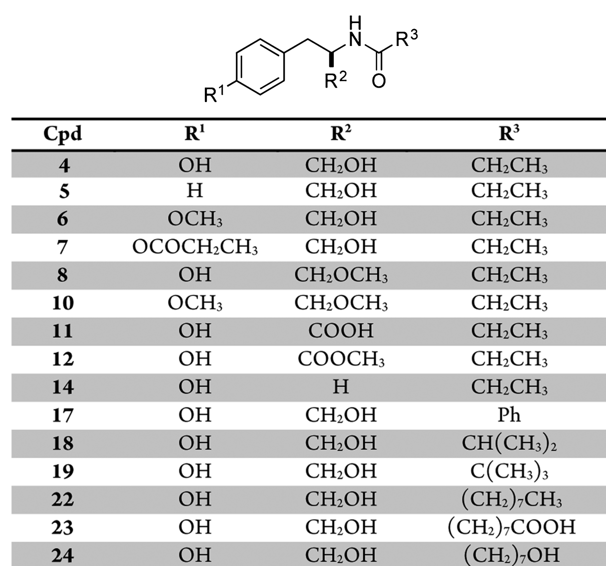
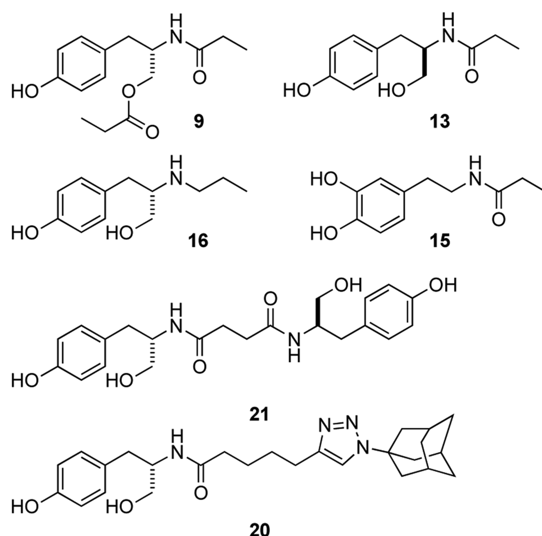
very limited structure–activity relationship study (SAR), we learned that the much simpler L-tyrosinol-propionamide **4** was able to induce neuronal differentiation, albeit at higher concentration than the parent analog **3**.[22] We therefore aimed to synthesize structurally optimized derivatives of farinosone C (**3**), which retain or even display more potent neurite outgrowth inducing capability. At the same time, this endeavor would also result in a more defined description of the pharmacophore. In this communication, we report the results of this SAR study on farinosone C (**3**) analogs and perform preliminary studies on the mechanism of action, identifying the modulation of the endocannabinoid system as a potential target pathway.

We prepared a collection of farinosone C (**3**) derivatives (Table 1; for details see Supporting Information) and profiled their neuritogenic properties (Figure 2) using a modified procedure of the well established PC12 cell assay that can serve as a simplified model system for NGF-mediated, neuronal differentiation.[21,22,26−28] First, the role of the phenolic OH group was evaluated. Since phenols are known to be easily oxidized in an enzymatic environment,[29] we envisioned that the removal (→ **5**), methylation (→ **6**), or esterification (→ **7**) of the hydroxyl group might be beneficial for metabolic stability. However, all these modifications led to a complete loss of bioactivity, which suggested that this functional group is essential for activity. The role of the primary hydroxyl group was investigated next. Again, methylation and esterification were not tolerated, as the corresponding compounds **8** and **9** were found to be inactive, as was the doubly methylated compound **10**. Interestingly, however, oxidation to the carboxylic acid **11** and derivatization to its methyl ester analog **12** led to biologically active compounds. We then investigated the influence of the stereogenic center: formation of the D-tyrosinol-propionamide **13** or complete removal of the CH_2OH side chain in **14** resulted in inactivity, showing that the presence of the hydroxymethylene moiety in the (*S*)-configuration is mandatory for neuritogenic activity in the PC12 cell assay. The close catechol analog of **14**, dopaminyl propanoate (**15**), caused cytotoxicity at our standard concentration of 50 μM and was inactive at 5 μM. The amide moiety was also shown to be essential, as the secondary amine **16** was inactive. Modifications of the amide only tolerated a planar aromatic substituent (→ **17**); the more bulky isobutyramide **18** and pivalamide analogs **19** displayed no significant activity. With these results in hand, we turned our attention toward the alkyl chain, the part of farinosone C (**3**) that has synthetically been the most demanding. The very bulky triazole-adamantyl derivative **20** was not active; however, the dimer **21** of L-tyrosinol-propionamide **4** showed significant bioactivity. Finally, we were interested in the role of the acidic terminus of farinosone C (**3**). It appears that a polar terminus is required for the long chain aliphatic compounds, as the apolar amide **22** revealed no activity. Terminal acid **23** and the terminal alcohol **24** showed good activity; the latter compound was even able to induce cell differentiation at 10 μM concentration, thus rendering the triol **24** an even more potent compound than the parent natural product farinosone C (**3**, Figure 3). This interesting result demonstrated that the synthetically challenging side chain of the parent natural product can be replaced by an unbranched and fully saturated alkyl chain.

Table 1. Synthesized Farinosone C Analogs 4−24



Cpd	R¹	R²	R³
4	OH	CH_2OH	CH_2CH_3
5	H	CH_2OH	CH_2CH_3
6	OCH_3	CH_2OH	CH_2CH_3
7	$OCOCH_2CH_3$	CH_2OH	CH_2CH_3
8	OH	CH_2OCH_3	CH_2CH_3
10	OCH_3	CH_2OCH_3	CH_2CH_3
11	OH	COOH	CH_2CH_3
12	OH	$COOCH_3$	CH_2CH_3
14	OH	H	CH_2CH_3
17	OH	CH_2OH	Ph
18	OH	CH_2OH	$CH(CH_3)_2$
19	OH	CH_2OH	$C(CH_3)_3$
22	OH	CH_2OH	$(CH_2)_7CH_3$
23	OH	CH_2OH	$(CH_2)_7COOH$
24	OH	CH_2OH	$(CH_2)_7OH$



Many natural products with neuritogenic or neuritotrophic properties have been reported.[4−11] However, the underlying biological pathways involved in neuronal cell differentiation, which are influenced by such compounds, are only partially understood. We therefore decided to investigate the molecular targets of the synthetic derivatives prepared that display neuritogenic properties. Computational approaches by structural similarity searching[30] hinted at fatty acid amides such as OMDM-2 (**25**) and OMDM-4 (**26**), which bind to the cannabinoid CB_1 receptors in the low micromolar range. These compounds serve as aromatic structural analogs for the endogenous cannabinoid *N*-arachidonoylethanolamine (AEA) **27** (Figure 4).[31−33]

Figadère and co-workers demonstrated that fatty acid amide derivatives of tryptamine are also able to induce neuronal differentiation.[34] Activation of the CB_1 receptor was reported to induce neuronal differentiation through a complex signaling network in Neuro-2A cells[35,36] and to restore the neurite outgrowth in hyperglycemic PC12 cells.[37] Based on these structural precedents for both CB_1 binding and its role as neuritogenic target, we prepared a second collection of compounds (Table 2) by structurally expanding the heptamethylene scaffold of compounds **22**−**24** to obtain longer alkyl chains with no or partial unsaturation.

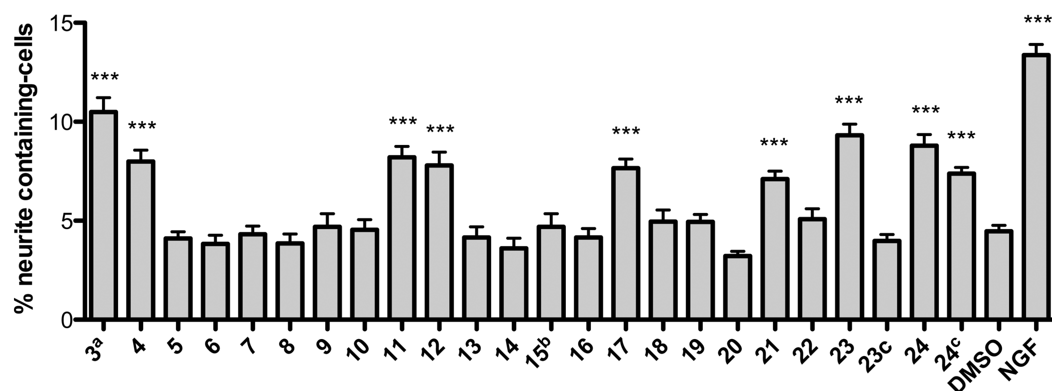


Figure 2. Neuritogenic activity of farinosone C (**3**) and its simplified analogs (**4**−**24**) in the PC12 assay. All values were determined at 50 μM, except the following: a, 20 μM; b, 5 μM; c, 10 μM. Positive control: nerve growth factor (NGF), 20 ng mL^{-1}. Solvent control: DMSO (0.1%). Incubation period: 2 days. Values are reported as mean (unpaired *t* test; confidence interval 95%; significance: *** = $P < 0.0001$, $n = 3$, mean ± SEM).

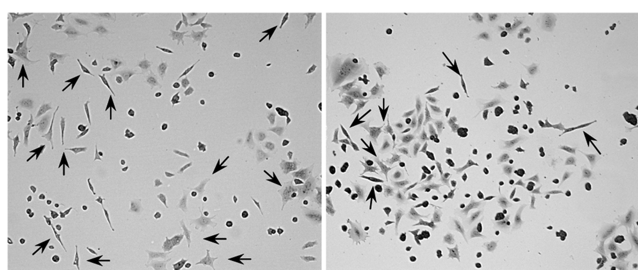


Figure 3. Representative images of differentiated PC12 cells: left, **24**, 10 μM; right, DMSO vehicle control. Arrows indicate differentiated PC12 cells.

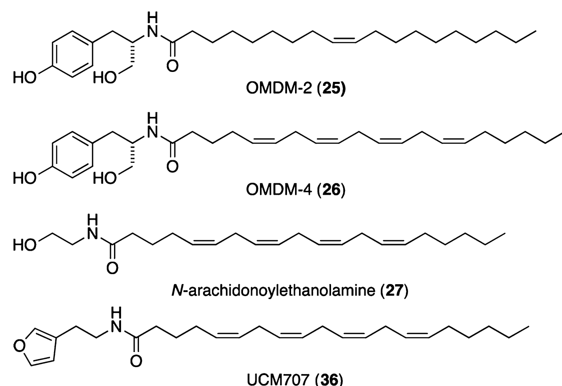


Figure 4. Chemical structure of the endocannabinoid AEA (**27**), two endocannabinoid-like CB_1 ligands (**25** and **26**) and the endocannabinoid transport inhibitor UCM707 (**36**).

Table 2. Fatty Acid Amide Derived Analogs 28−35

Cpd	X	R
28	–CH_2–	–$(CH_2)_7$–CH_3
29	–CH_2–	–$(CH_2)_7$–OH
30	–CH_2–	–CH=CH–CH_2–CH=CH–$(CH_2)_4$–CH_3
31	–CH_2–	–$(CH{=}CH{-}CH_2)_3$–CH_3
32	–C=O	–$(CH_2)_7$–CH_3
33	–C=O	–$(CH_2)_7$–OH
34	–C=O	–CH=CH–CH_2–CH=CH–$(CH_2)_4$–CH_3
35	–C=O	–$(CH{=}CH{-}CH_2)_3$–CH_3

Compounds **28**−**35** and OMDM-2 (**25**) were first screened for neurite outgrowth at 10 μM concentration. We noticed that all compounds bearing a secondary amine function (**28**−**31**) were not tolerated by PC12 cells, as they induced cytotoxicity. After reduction of the concentration by one order of magnitude, neither toxicity nor neuritogenic activity could be observed for these amines (Figure 5). Among the amide bearing amphiphiles (**25**, **32**−**35**), only **34** (hereafter referred as **BSL34**) showed a significant activity at 10 μM (Figure 5), which suggests that the degree of unsaturation of the alkyl chain plays a crucial role. Also the length of the alkyl chain is of importance; **24** has been identified as the most potent compound, whereas triol amide **33** is inactive. Interestingly, they only differ in the length of the alkyl chain (C7 versus C14).

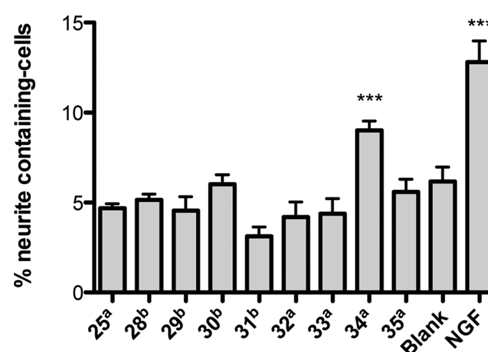


Figure 5. Neuritogenic activity of OMDM-2 (**25**) and fatty acid derived analogs. Values were determined at a: 10 μM, b: 1 μM, NGF control: 20 ng mL^{-1}. DMSO control: 0.1%. Incubation period: 2 days. Values are reported as means ± SEM (Unpaired *t* test, confidence interval 95%, significance: *** = $P < 0.0001$, $n = 3$).

Next, we investigated the binding affinities for the compounds at CB receptors using membrane preparations from CHO cells stably transfected with human CB receptors. Most of the compounds showed weak binding to CB_1 and CB_2 receptors at the screening concentration of 1 μM (Supporting Information Figure 1A and 1B). Nonetheless, we noticed that **29** and, more importantly, the neuritogenic omega-6 fatty acid amide derivative **BSL34** selectively bind to the CB_1 receptor with moderate potency (K_i values of 530 and 810 nM, respectively (Figure 6)).

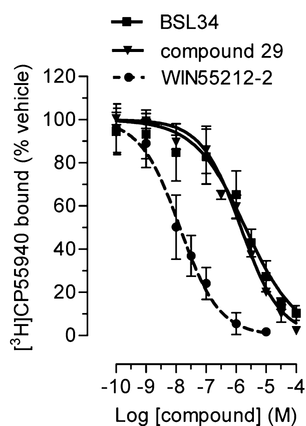


Figure 6. CB_1 binding properties of compounds **29** and **BSL34**. The assay was performed as described before[38] by incubating for 90 min at 30 °C CHO-*h*CB1 membranes with different concentrations of tested compounds (or the positive control, WIN55,212-2) in the presence of 0.5 nM [^{3}H]-CP55940 (mean ± SD; *n* = 2−3).

To further clarify the role of the CB_1 receptor in our system, we incubated PC12 cells with the CB_1 selective agonist O-689 at 100 nM and with two CB_1 antagonists, rimonabant (1 μM and 100 nM) and AM251 (3 μM and 100 nM).[39,40] Despite multiple attempts under manifold conditions, neuronal differentiation could not be achieved by O-689 treatment nor were the selective CB_1 antagonists able to suppress the **BSL34**-induced neuronal differentiation, suggesting that CB_1 is not directly involved in our PC12 neurite outgrowth assay. The endocannabinoid system included several proteins involved in the biosynthesis, degradation, and trafficking of the two main endogenous ligands of CB receptors, AEA (**27**), and 2-arachidonoyl glycerol (2-AG). Intra- and extracellular levels of 2-AG and AEA (**27**) are under control of degrading enzymes, intracellular carriers, and the putative endocannabinoid membrane transporter (EMT). Modulation of those targets' function leads to a change in the levels of AEA (**27**) and 2-AG, thus raising indirect CB receptor activation.[41] We have therefore evaluated the impact of our compounds on the activity of those targets. Most of the compounds tested at 1 μM showed a weak inhibition (20−25%) of fatty acid amide hydrolase (FAAH), the main enzyme involved in AEA hydrolysis (Supporting Information Figure 2). The main enzymes involved in 2-AG hydrolysis (monoacylglycerol lipase, MAGL, α,β-hydrolase-6 and -12, ABHD-6 and -12) were not significantly inhibited at the same concentration (Supporting Information Figure 3A and 3B). AEA analogs (**28**−**35**) could also modulate the trafficking of endocannabinoids across the plasma membrane. Gratifyingly, this hypothesis was corroborated by the discovery of four fatty acid amides as excellent EMT inhibitors. Four of the tested compounds inhibited AEA (**27**) cellular uptake with submicromolar EC_{50} values. The most potent compound, **BSL34**, inhibited the EMT activity with one order of magnitude more than UCM707 (**36**), a commercially available benchmark inhibitor (Table 3 and Supporting Information Figure 4).[41−43]

With the synthesized collection of farinosone C analogs reported herein, we were able to elucidate the structure requirements for activity derived from the parent natural product **3**. It was demonstrated, for example, that the branched and unsatured side chain can by simplified or truncated. The phenolic hydroxyl group allowed no alteration, but the primary

Table 3. [^{3}H]AEA transport inhibition parameters. EC_{50} values and maximal effect were determined from concentration-dependent curves performed in U937 human leukemic monocyte lymphoma cells as previously described (*n* = 3-5).[41]

Cpd	EC_{50} ± SEM (nM)	Max inhibition
30	975 ± 201	51 %
31	966 ± 252	60 %
33	757 ± 326	73 %
BSL34	228 ± 139	52 %
UCM707	1800 ± 801	80 %

one did to some extent. This SAR study unearthed seven neuritogenic molecules (**10**, **11**, **17**, **21**, **23**, **24**, **BSL34**), of which two, the triol **24** and the fatty acid derivative **BSL34**, possessed a superior neurotrophin-like function than the natural product **3** itself, with a much reduced molecular complexity. Both can be obtained from cheap commercial starting materials in one step, and supply is therefore ensured. Our data also suggest the involvement of the endocannabinoid system in neuronal differentiation induced by these classes of compounds. The previously reported[44] CB_1 receptor-induced neuritogenic effect was not reproduced in our hands, as the selective agonist O-689 did not lead to any significant neuronal differentiation and the **BSL34**-induced effect was not blocked by the selective CB_1 receptor antagonists AM251 or rimonabant. The experimental conditions of the PC12 assay have a significant impact on the read-out. Indeed, HU-210 was shown to restore the neurite outgrowth in hyperglycemic cells to a degree comparable with normal cells in a CB_1 receptor-dependent mechanism, while contrasting data were reported for normoglycemic cells. CB_1 receptor activation was shown either to trigger[44] or to impair neurite outgrowth.[45] In addition, different studies describe a variable CB_1 receptor expression in PC12 cells. The receptor was either found[37] or not found[46,47] on the plasma membrane of undifferentiated PC12 cells. Others reported CB_1 receptor expression only in NGF-differentiated PC12 cells.[45] Possible reasons for apparent discrepancies between these and our findings might relate to the relative expression level of CB_1 receptors and thus point toward a CB_1 receptor-independent effect. However, as **BSL34** showed a potent EMT inhibition, we assume that the resulting changes in the local concentration of endocannabinoids, for example, AEA and 2-AG, could affect cell differentiation. EMT is involved in the bidirectional trafficking of endocannabinoids across the plasma membrane,[41] and its inhibition leads to a different compartmentalization of AEA (**27**) and 2-AG. AEA (**27**) has several other targets beyond the plasma membrane located $CB_{1/2}$ receptors, such as TRPV1 channels, intracellular CB_1 receptors, and nuclear PPARs. Some of those receptors are involved in neuronal differentiation. For example, PPAR-γ activation was shown to induce neurite outgrowth in SH-SY5Y human neuroblastoma cells,[48] while TRPV1 is involved in maintaining the [Ca^{2+}] homeostasis which is primarily involved during the development and differentiation of the nervous system. TRPV1 expression and activity was found to be increased in SH-SY5Y upon neuronal differentiation.[49] TRPV1 was also shown to be functionally expressed in PC12 cells,[50] which also are able to synthesize AEA (**27**).[51] Therefore, TRPV1 might be one of the candidates for the AEA-induced CB_1 receptor-indpendent targets of neurite outgrowth shown in

our report. The EMT inhibition could be the main mechanism of the neuritogenic effect shown by **BSL34**. The other bioactive compounds (**10**, **11**, **17**, **21**, **23**, and **24**) still inhibit the EMT, although with a lower potency (20−25% inhibition of AEA (**27**) uptake at the screening concentration of 10 μM (Supporting Information Figure 4) and only show moderate FAAH inhibition (Supporting Information Figure 2).

In conclusion, our results suggest that the modulation of the endocannabinoid transport could be the main mechanism of farinosone C and analogs with respect to the neuritogenic effects. Nevertheless, further investigations on the precise involvement of the endocannabinoid system in neuronal differentiation in PC12 cells will be performed to clarify the observed biological effects.

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental details and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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