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Stolonidiol: Synthesis, Target Identification, and Mechanism for Choline Acetyltransferase Activation

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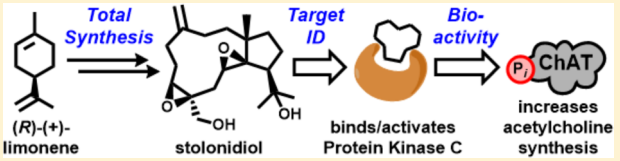
Supporting Information

ABSTRACT: Stolonidiol, a marine natural product, has been reported to potentiate the activity of choline acetyltransferase (ChAT), the enzyme that produces the neurotransmitter acetylcholine. Here we report the total synthesis of stolonidiol starting from (*R*)-(+)-limonene. To identify the mechanism by which ChAT activity is increased, we sought to identify the biological target of stolonidiol. We show that stolonidiol binds to the phorbol ester binding site of protein kinase C (PKC), induces translocation of PKC to the cell membrane, and activates kinase activity. Furthermore, we confirmed the increase in ChAT activity observed upon treatment of cells with stolonidiol and show that this effect is mediated by PKC. Collectively, our data strongly suggest that PKC activation by stolonidiol is responsible for the resulting potentiation of ChAT activity.



INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and currently afflicts 5.4 million Americans.[1] Alarmingly, current projections indicate that the prevalence of this disease will nearly triple over the next 35 years.[2] Although the mechanistic details remain obscure, cholinergic dysfunction appears early in the course of AD and leads to severe reductions in cholinergic signaling.[3] To counteract these losses, acetylcholinesterase (AChE) inhibitors are used to prevent the breakdown of acetylcholine and thus enhance cholinergic neurotransmission. Indeed, three of the four FDA approved treatments for AD are AChE inhibitors, which have been the first-line therapies for over two decades.[4]

As an alternative to preventing the breakdown of acetylcholine, a complementary approach for enhancing cholinergic signaling could be the activation of acetylcholine synthesis (Figure 1). For example, if a small molecule could be identified that enhanced choline acetyltransferase (ChAT) activity, the enzyme responsible for acetylcholine synthesis, enhancements in cholinergic signaling might be realized. Dramatic reductions in ChAT activity have been observed in AD patients,[5] and combining therapies that prevent neurotransmitter breakdown *and* amplify neurotransmitter synthesis may prove beneficial.

Stolonidiol (**1**), a natural product isolated from a Japanese soft coral,[6] has been reported to enhance ChAT activity.[7] Given the potential therapeutic benefits of enhancing ChAT activity, we became interested in identifying the mechanism by which **1** is able to produce this effect. We first developed a synthetic route to access the natural product and subsequently surveyed candidate cellular targets that might account for the reported increase in ChAT activity. We find that activation of protein kinase C (PKC) is the likely mechanism for the observed increase in ChAT activity.

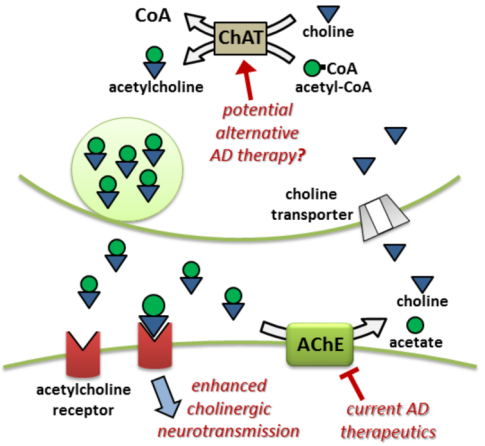


Figure 1. Cholinergic neurons are especially sensitive to AD pathology. Current therapeutics target acetylcholinesterase (AChE), preventing the breakdown of neurotransmitter and thereby increasing cholinergic signaling. Strategies aimed at increasing the production of acetylcholine via the activation of choline acetyltransferase (ChAT) may provide a synergistic approach to potentiating cholinergic neurotransmission.

There is currently a single total synthesis reported for **1** that required 36 steps.[8] The published synthesis employed a Horner–Wadsworth–Emmons (HWE) macrocyclization to form the 11-membered ring, which was followed by selective epoxidations to complete the natural product (Scheme 1). We hoped to develop a more expedient route to **1**, but chose to adopt the HWE ring closing strategy and targeted **3** as our key intermediate. Proceeding retrosynthetically from **3**, we felt that

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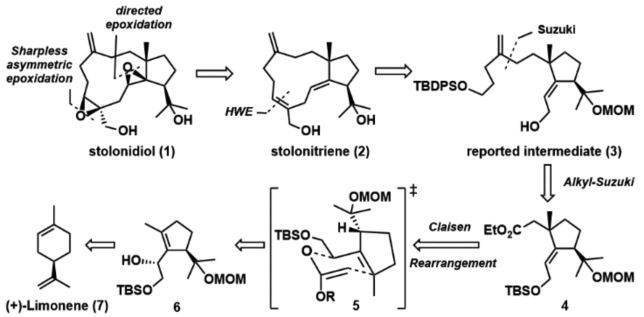
Scheme 1. Retrosynthesis of Stolonidiol (1)

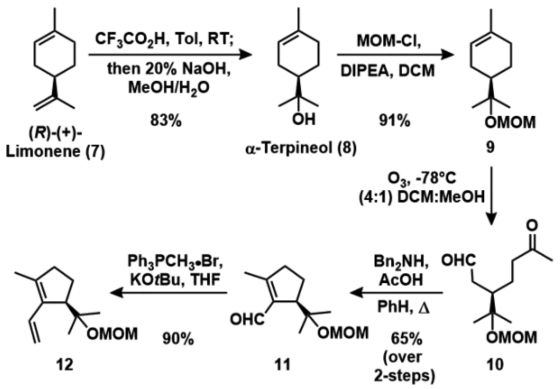


the pendant disubstituted alkene might be disconnected via alkyl-Suzuki coupling of an intermediate derived from **4**. We believed that the all-carbon quaternary stereocenter of **4** could be installed through a Claisen rearrangement via the cyclic chairlike transition state depicted in **5**. Finally, we anticipated that allylic alcohol **6** would be accessible from readily available (*R*)-(+)-limonene (**7**).

SYNTHESIS OF STOLONIDIOL

Our synthesis began with hydration of the exocyclic alkene of **7** using a procedure previously reported for the kilogram scale production of fragrance chemical α-terpineol (**8**) (Scheme 2).[9]

Scheme 2. Conversion of Limonene into Diene 12

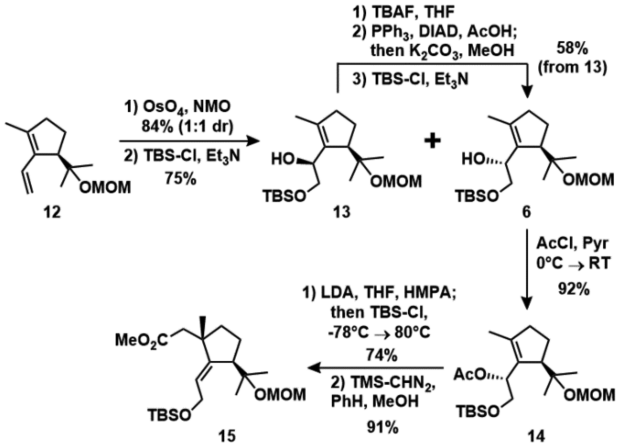


Methoxymethyl ether (MOM) protection of the resulting tertiary hydroxyl produced **9**, and then the remaining endocyclic olefin was cleaved by ozonolysis. Next, we sought to cyclize keto-aldehyde **10** to cyclopentenone **11** using an enamine-based cyclization approach. While analogous cyclizations have been reported for substrates lacking the tertiary hydroxyl,[10−12] this additional functionality blocked the production of **11** using piperidine or pyrrolidine catalysis. Screening a variety of amine and acid catalysts led to the identification of dibenzylamine and acetic acid as the optimal catalyst combination (Supporting Information Table S1). Wittig olefination of aldehyde **11** then provided diene **12** in 90% yield.

At this point, we hoped to perform a regio- and diastereoselective dihydroxylation of **12**. We anticipated that if the diene system adopted a planar *s*-trans conformation, then dihydroxylation from the top face of the exocyclic olefin would be blocked by the MOM-protected isopropoxy substituent, which would direct dihydroxylation to the bottom face of this olefin. Surprisingly, dihydroxylation of **12** produced a 1:1 mixture of diol diastereomers which were inseparable by column chromatography. To overcome this lack of selectivity, we screened a variety of dihydroxylation conditions, including several asymmetric methods.[13−15] Unfortunately, we were unable to identify conditions that provided useful levels of selectivity in this system.

As an alternative, we developed a procedure for converting the undesired diol diastereomer into the desired one (Scheme 3). First, selective *tert*-butyl dimethylsilyl ether (TBS)

Scheme 3. Conversion of Dihydroxylation Products into Desired Isomer 6 and Initial Ireland−Claisen Rearrangement



protection of the primary hydroxyls provided a mixture of **13** and **6**, which were separable by chromatography. Identification of the hydroxyl configuration was established using Mosher ester analysis[16] of each isomer (see Supporting Information Figure S1). Unfortunately, **13** could not be converted directly to **6**, presumably due to steric hindrance around the secondary hydroxyl. We eventually found that deprotection of the TBS group of **13**, followed by Mitsunobu inversion of the free diol[17] and reprotection of the TBS group provided a three-step sequence that afforded **6** in 58% yield from **13**.

With **6** in hand, we began exploring the use of an Ireland−Claisen rearrangement[18] to generate the required quaternary stereocenter. Acetylation of **6** provided **14**, which was converted into the silyl enol ether derivative by treatment with LDA in THF−HMPA and TBS−Cl at −78 °C. This solution was then slowly warmed to 80 °C (for 16 h) to promote the Claisen rearrangement. Using this procedure, we were able to isolate the carboxylic acid corresponding to **15** in 74% yield as the only isomer detected in the reaction mixture. Esterification of the carboxylic acid with trimethylsilyl diazomethane then provided **15** in 91% yield.

While the Ireland−Claisen procedure provided efficient access to intermediate **15**, we wondered if this three-step sequence (acetylation, rearrangement, and esterification) could be reduced to a single-step by employing a Johnson−Claisen protocol.[19] We were initially leery of the high temperature and acidic conditions required for this rearrangement, but to our delight, we found that treatment of **6** with triethylorthoacetate and catalytic pivalic acid at 145 °C provided **4** in 74% yield on gram scale (Scheme 4). Ester **4** was further processed to give alkyl-iodide **16** in 91% yield over two steps.

Coupling of **16** with vinyl iodide **17** using the alkyl-Suzuki conditions developed by Fürstner and colleagues[20] provided the desired product, which was directly deprotected to give

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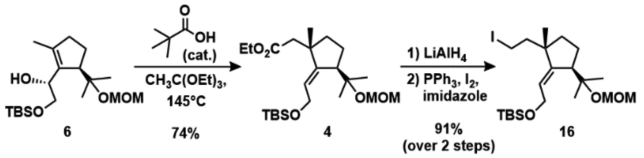
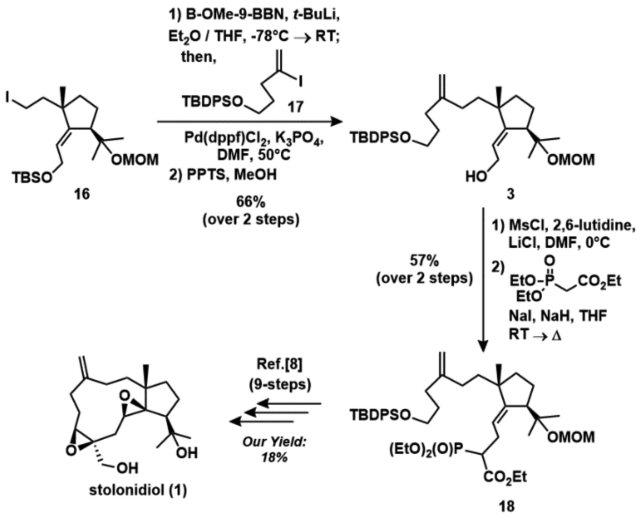
Scheme 4. Johnson–Claisen Rearrangement and Conversion to Coupling Precursor 16



known intermediate **3** and complete a formal total synthesis (Scheme 5). With intermediate **3** in hand, we were able to

Scheme 5. Completion of the Synthesis of 1



complete the synthesis of **1** using the previously reported route,[8] with one notable modification. In our hands, the reported one-step conversion of **3** to **18** provided inconsistent results (see Supporting Information). We developed a two-step procedure that begins with allylic chlorination of **3** using the procedure of Meyers,[21] followed by phosphonate displacement of an *in situ* formed allylic iodide providing intermediate **18** in 57% yield over the two steps. Full experimental details for the conversion of **3** to **1** are provided in the Supporting Information.

BIOLOGICAL TARGET IDENTIFICATION

After evaluating the various pathways that have been reported in the literature to enhance ChAT activity, we identified PKC as a promising potential target. Most notably, the Rylett laboratory has reported a series of studies that identified ChAT as a substrate for PKC-mediated phosphorylation, which was further shown to modulate ChAT activity.[22−24] We performed docking studies that suggested that the phorbol ester binding site of PKC[25] may accommodate stolonidiol, adding plausibility to our hypothesis that PKC could be a biological target of **1** (Figure 2).

To test this hypothesis, we began by performing a radioligand displacement assay with PKCα and [^{3}H]-phorbol dibutyrate (PDBu) (Figure 3).[27] Addition of increasing concentrations of unlabeled PDBu or **1** both displaced the radioligand and gave IC_{50} values of 39 nM and 3.1 μM, respectively, suggesting that **1** does in fact interact with the phorbol ester binding site of PKC, although less potently then PDBu.

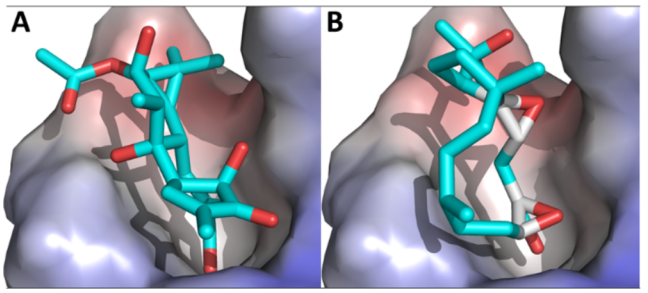


Figure 2. (A) Crystal structure of a phorbol ester bound to PKC (PDB: 1PTR). (B) Possible binding model of **1** docked in the phorbol ester binding site of PKC using AutoDock Vina.[26]

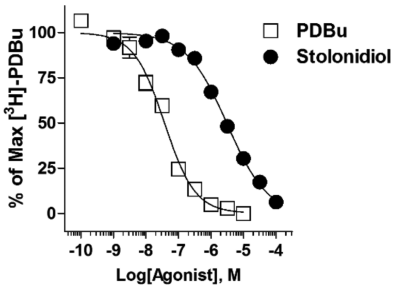


Figure 3. PKCα radioligand displacement assay using [^{3}H]-PDBu. Maximum displacement represents 10 μM cold PDBu and % displacement curves are shown for cold PDBu (IC_{50} = 39 nM) and **1** (IC_{50} = 3.1 μM). Data points were run in triplicate (±SEM).

Prior to activation, PKC translocates to the cell membrane where further structural rearrangements result in kinase activation.[28] As such, translocation to the cell membrane provides a well-established marker of PKC activation. To investigate whether binding of **1** to PKC leads to kinase activation, we next performed a translocation assay using a green-fluorescent protein (GFP)-tagged PKC (Figure 4).[29] Following transfection of a PKC-GFP plasmid into HEK293 cells, the labeled protein was found distributed throughout the

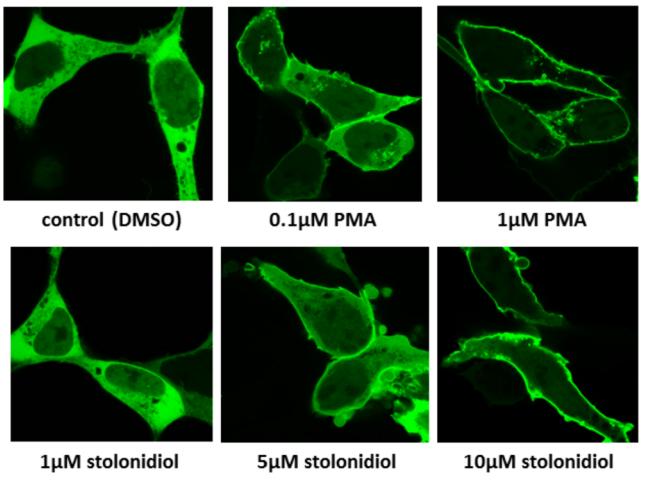


Figure 4. GFP-labeled PKCγ was transfected in HEK293 cells, and cells were treated with PMA or stolonidiol at the indicated concentrations. Vehicle treated cells show PKC-GFP distributed throughout the cytosol, whereas treatment of cells with 1 μM PMA and 10 μM **1** produces pronounced translocation to the plasma membrane. Treatment with 0.1 μM PMA and 5 μM **1** induced partial translocation; 1 μM **1** did not induce translocation.

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cytosol and addition of vehicle produced no change in PKC localization (Figure 4). However, when cells were treated with 1 μM phorbol 12-myristate-13-acetate (PMA), or 10 μM **1**, we observed robust translocation of the labeled protein to the cell membrane. Treating cells with 0.1 μM PMA or 5 μM **1** induced a partial response, whereas 1 μM **1** did not induce translocation. Therefore, treatment with **1** is able to induce PKC translocation to the cell membrane, indicative of kinase activation.

Upon activation, PKC phosphorylates a consensus sequence that is found on multiple cellular proteins. To further examine the ability of **1** to induce activation of PKC, a commercially available monoclonal antibody mixture (MultiMab) that specifically recognizes PKC consensus sequences containing a phosphorylated serine was used to assess kinase activity. We treated HEK293 cells with 1 μM PMA, 1 μM **1**, or 10 μM **1** and performed a Western blot analysis of cell lysates (Figure 5).

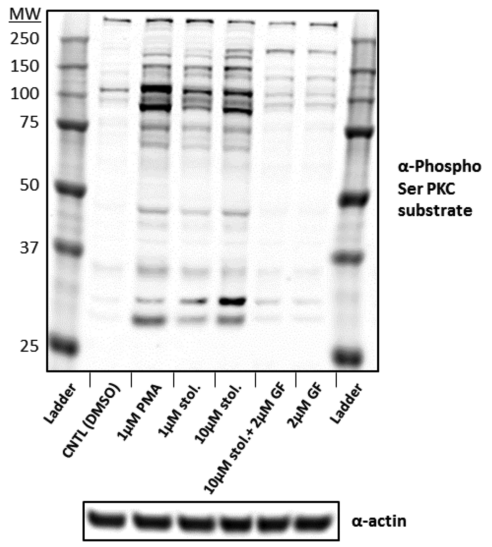

Figure 5. HEK293 cells were treated for 20 min with DMSO (control), 1 μM PMA, 1 μM **1**, or 10 μM **1**, and cell lysates were probed with antibody specific for substrates of PKC that have been phosphorylated at a serine residue within the PKC consensus sequence. Cotreatment of cells with 10 μM **1** and 2 μM GF109203X (GF) reverses the increase in substrate phosphorylation, whereas treatment with 2 μM GF alone does not affect substrate phosphorylation.

In comparison to control (DMSO) treated cells, those treated with PMA or with **1** showed a strong increase in substrate phosphorylation, further supporting the notion that **1** is able to activate PKC in cells. Cotreatment of cells with 10 μM **1** and 2 μM GF109203X (GF), a bis-indolylmaleimide inhibitor of PKC,[30] reversed the observed increase in substrate phosphorylation indicating that PKC activation is mediating phosphorylation.

Finally, having generated strong evidence in support of the ability of **1** to activate PKC, we wanted to confirm that treatment of cells with **1** resulted in increased ChAT activity. We first generated stably transfected ChAT expressing HEK293 cells and confirmed ChAT expression via immunofluorescence (Supporting Information Figure S2). We then treated these cells with 1 μM PMA and measured ChAT activity using the method of Fonnum,[31] after 16 h of treatment. In agreement with previous reports,[23] we observed a 61% increase in ChAT activity over control treated cells (Figure 6). We next examined ChAT activity following treatment with **1** and found that 1 and

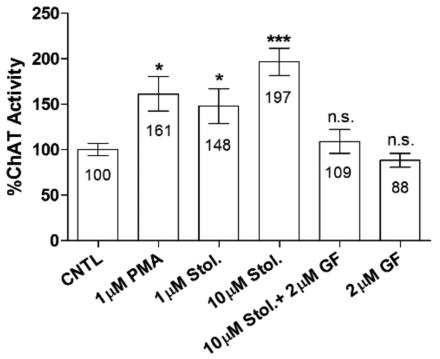

Figure 6. HEK293 cells stably expressing 69 kDa ChAT were treated as indicated for 16 h, and ChAT activity (relative to control) was measured in crude cell lysates. Data are expressed as mean ± SEM of five experiments. Statistical significance (Student's *t*-test) versus control: * $p < 0.05$; *** $p < 0.0005$; n.s. = not significant.

10 μM treatments produced 48% and 97% increases in ChAT activity, respectively. To confirm that this increase in ChAT activity was mediated by PKC, we cotreated cells with 10 μM **1** and 2 μM GF. Cotreatment with the PKC inhibitor reversed the observed increase in ChAT activity, suggesting that the increased activity is mediated by PKC.

Reduced PKC activity has been observed in AD patients, and PKC activity is known to play a crucial role in memory formation.[32,33] The culmination of these effects has led to the hypothesis that PKC activators may provide symptomatic benefits in AD. Indeed, the potent PKC activator bryostatin 1 is currently being investigated as a potential AD therapeutic.[33−36] We show here that an additional benefit of PKC activation may arise from subsequent ChAT activation, adding further promise to this approach.

CONCLUSIONS

In summary, we describe the use of the natural product stolonidiol to probe pathways responsible for activating neurotransmitter biosynthesis. Similar mechanistic investigations of small molecules that enhance neurotransmission may provide a general approach for identifying therapeutic targets for complex neurodegenerative diseases.

EXPERIMENTAL SECTION

For experimental details, see Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b01083.

Experimental details and procedures (PDF)
Spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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