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Neurosteroids

# Synthesis of Withanolide A, Biological Evaluation of Its Neuritogenic Properties, and Studies on Secretase Inhibition**

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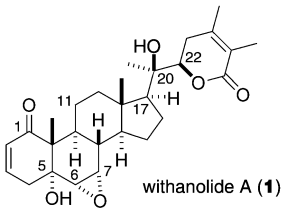
The enhancement of memory and learning by small molecules is of significant current interest to society.[1] This approach could alleviate the course of neurodegenerative diseases such as Alzheimer's or Parkinson's.[2] In addition, the use of cognitive enhancers by healthy subjects for increased intellectual performance is under intense current debate.[1,3] For both aspects, traditional medicinal plants constitute a valuable resource, in particular regarding the identification and synthesis of biologically active lead structures.[4] *Withania somnifera* ("Ashwagandha" in Ayurveda, Indian ginseng) is considered an important plant in traditional Indian medicine and is being prescribed for a variety of ailments, including as a general tonic for the elderly related to antiaging and cognitive improvement.[5] Molecular pharmacological studies have associated some of these activities with certain metabolites in the plant. In particular, withanolide A (**1**) has been shown to possess strong neuropharmacological activities in promoting neurite outgrowth, reversing neuritic atrophy, and aiding synapse reconstruction.[6] These remarkable properties are further extended by a recent study documenting that withanolide A (**1**) modulates several secretase targets with regard to neurodegenerative diseases.[7] Chan and co-workers recently demonstrated that this steroid lactone **1** downregulates BACE1 and upregulates ADAM10 in primary rat cortical neurons.[7] These combined reports provide a strong pharmacological rationale for further investigation of withanolide A (**1**), for which no preparation from simple steroid precursors has yet been reported.[8]

Herein, we report the first successful synthesis of withanolide A (**1**) from pregnenolone featuring a singlet $O_2$ ene reaction, a vinylogous aldol reaction, and a Wharton carbonyl transposition. In addition, we have re-evaluated the neuritogenic properties of **1** and of its derivatives and have also carried out mechanism-of-action studies using a series of enzymatic assays.




withanolide A (**1**)

The steroid lactone withanolide A (**1**) displays functionalities in both the A and the B ring, as well as the functionalized side-chain lactone, which raise strategic ramifications concerning the sequential order of their installation. In particular, the oxidation pattern at C5–C7 as well as the tertiary alcohol at C20 combined with the adjacent dihydropyrone were identified in the beginning as key challenges for the synthesis. During semi synthesis studies to derivatize withanolide A, it became clear that both the side chain as well as the hydroxy epoxide in the B ring are relatively stable under a variety of conditions. Surprisingly, after extensive experimentation both on model systems as well as on the natural product, we realized that the chemically most sensitive functionality was the α,β-unsaturated ketone in the A ring. By building on this chemical knowledge and hoping to exploit the inherent reactivity pattern of withanolide A (**1**), we planned to minimize the use of protecting groups towards the end of the synthesis. Therefore, we decided to stereoselectively install the dihydropyrone carbinol group first, with subsequent oxidation of the B ring and finally installation of the enone in the A ring.

The synthesis of withanolide A (**1**) started with the protection of the hydroxy group of pregnenolone as a TBS-ether; the keto group was then homologated using the Corey–Seebach umpolung methodology to provide known[9] dithiane **2** in 82% yield over two steps (Scheme 1). The oxidative cleavage of the 1,3-dithiane by *N*-chlorosuccinimide proceeded smoothly to provide the corresponding $C_{22}$ hydroxy aldehyde, of which the tertiary C20-OH group was protected as MOM ether (94%). Following a procedure developed by Ikekawa,[8d] the resulting aldehyde **3** reacted with the vinylogous enolate (from ethyl 2,3-dimethylbut-2-enoate[10] and LiHMDS) in a stereoselective vinylogous aldol reaction to provide the lactone **4** in very good yield and stereoselectivity (87%, d.r. = 93:7). The configuration of both C20 with the tertiary hydroxy group and the newly formed stereogenic

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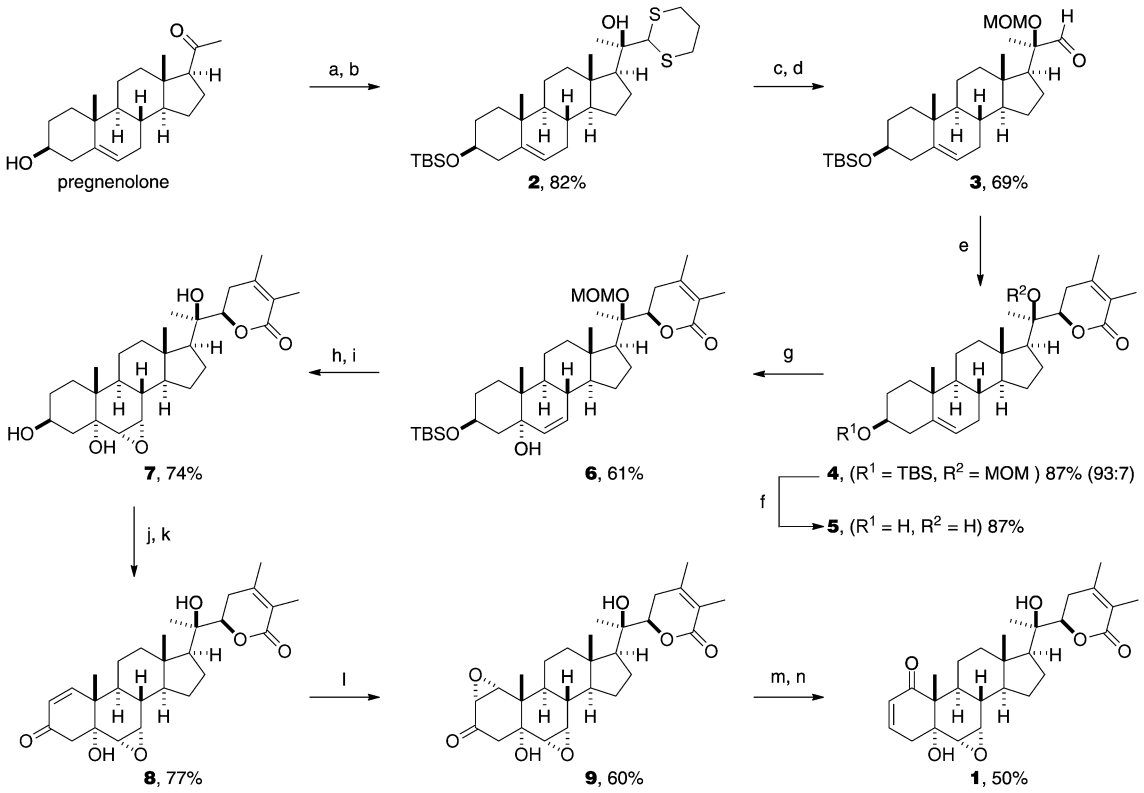



***Scheme 1.*** Total synthesis of withanolide A (**1**): a) TBSCl, Imd, THF, RT, 98%; b) dithiane, BuLi, THF, −78°C to RT, 84%; c) NCS, $CH_2Cl_2$, RT, 73%; d) MOMCl, NaI, DIPEA, DME, reflux, 94%; e) ethyl-2,3-dimethylbut-2-enoate, LiHMDS, THF/DMPU, −78°C to RT, 87% (93:7); f) HCl, THF/$H_2O$, RT, 87%; g) $O_2$, TPP, Na light, pyridine, $PPh_3$, RT, 61%; h) 3-chloroperbenzoic acid, $CH_2Cl_2$, 0°C to RT, 96% (96:4); i) HCl, THF/$H_2O$, RT, 80%; j) TPAP, NMO, $CH_2Cl_2$, RT, 95%; k) IBX, MPO, DMSO, 40°C, 81%; l) $H_2O_2$, Triton B, THF, 0°C, 60%; m) $N_2H_5Cl$, $Et_3N$, 0°C to RT, 62%; n) PDC, $CH_2Cl_2$, RT, 80%. TBSCl = *tert*-butyldimethylsilyl chloride, NCS = *N*-chlorosuccinimide, MOMCl = (chloromethyl)methyl ether, DIPEA = diisopropylethylamine, LiHMDS = lithium hexamethyldisilazide, DMPU = N,N′-dimethylpropyleneurea, TPP = *meso*-tetraphenylporphyrin, TPAP = tetrapropylammonium perruthenate, NMO = *N*-methylmorpholine *N*-oxide, IBX = 2-iodoxybenzoic acid, MPO = 4-methoxypyridine-N-oxide, Triton B = benzyltrimethylammonium hydroxide, PDC = pyridinium dichromate.

center were confirmed by X-ray crystal structure analysis of deprotected lactone **5** (Figure 1).

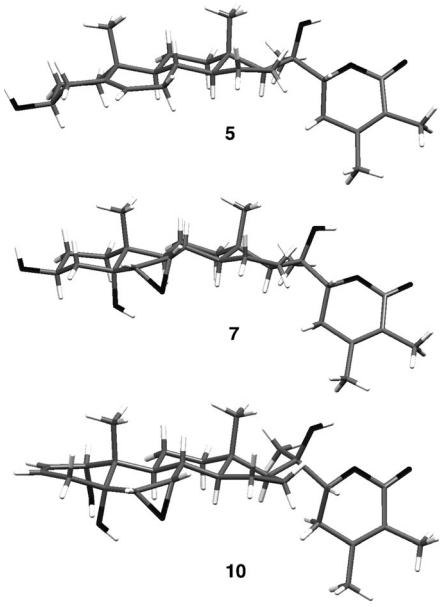



***Figure 1.*** X-ray crystal structures of compounds **5**, **7** and **10**.[19]

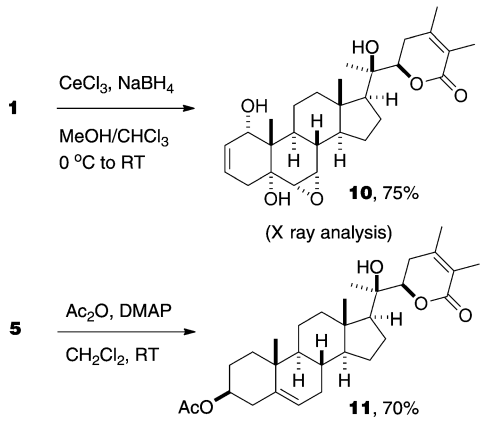
We then planned to install the correct oxidation pattern in the B ring of withanolide A (**1**). The challenge consisted of the regioselective installation of the B-ring epoxyalcohol from a simple olefin in **4** in the presence of the unsaturated lactone. After a thorough experimental investigation of various possibilities, we identified the singlet-oxygen-mediated photooxygenative olefin migration as a straightforward method[11] for the synthesis of the allylic tertiary alcohol **6**. Accordingly, the olefin **4** was allowed to react with singlet oxygen, generated in situ from $O_2$, in the presence of *meso*-tetraphenylporphyrin as sensitizer and under irradiation of a Na lamp, affording the allylic alcohol **6** in good yield (61%). 3-Chloroperbenzoic acid mediated stereoselective directed epoxidation of allylic alcohol **6** proceeded cleanly to provide the desired epoxyalcohol in excellent yield, which was further converted to triol **7** by treatment with aqueous HCl. The configuration of intermediate **7** was unambiguously established by X-ray crystal structure analysis (Figure 1).

After having established the correct functionalization of both the B ring and the dihydropyrone carbinol, we next addressed the elaboration of the A ring. In particular, the final steps from **7** were carried out without the use of protecting groups, thus exploiting the inherent reactivity pattern. Therefore, the challenge consisted of the correct

transformation of the A ring to the enone in the presence of two tertiary hydroxy groups. Oxidation of the secondary hydroxy group of triol **7** by tetrapropylammonium perruthenate (TPAP) and *N*-methyl morpholine *N*-oxide (NMO) provided the corresponding ketone. Further oxidation of this ketone to the enone **8** (81%) was mediated by 2-iodoxybenzoic acid (IBX) under the optimized conditions initially reported by Nicolaou, Montagnon, and Baran.[12] Other methods such as the Saegusa oxidation provided lower yields, and dichlorodicyanobenzoquinone did not result in the desired product. The enone **8** was epoxidized by aqueous hydrogen peroxide in the presence of triton B,[13] affording epoxy ketone **9** (60%) and setting the stage for the final key step of the synthesis: a Wharton carbonyl transposition[14] of **9**. Hence, compound **9** was treated with hydrazine hydrochloride in the presence of base to give the rearranged allylic alcohol. This reaction proceeded smoothly to furnish, after subsequent oxidation by pyridinium dichromate, withanolide A in good yield (50% over two steps). The analytical data ($^1$H and $^{13}$C NMR spectroscopy, MS, UV spectroscopy, melting point, HPLC analyses) for synthetic withanolide A (**1**) were found to be in full agreement with those of an isolated natural sample.[15] In addition, the identity of natural and synthetic samples was confirmed by mixing natural and synthetic samples and analyzing them by NMR spectroscopy and HPLC.

Derivatives of **1** were obtained either by semi-synthesis of the natural product (extracted and purified from the dried roots of *Withania somnifera*)[15,6a] or by further transformations of synthetic intermediates (Scheme 2). The challenge




***Scheme 2.*** Synthesis of A-ring-modified derivatives. DMAP = 4-(dimethylamino)pyridine.

with regard to semi-synthesis of **1** resides in its low solubility and the high reactivity of the A ring, which led to decomposition and undesired side reactions under a variety of conditions. After considerable experimentation, regioselective 1,2-reduction of the α,β-unsaturated ketone to the allylic alcohol **10** by Luche reduction[16] could be achieved in good yields. The configuration was established by X-ray crystal structure analysis (Figure 1). Interestingly, the configuration of the C1 stereogenic center in compound **10** was found to be identical to that obtained after the Wharton reaction. Acetylation of compound **5** under standard conditions led to the protected lactone **11**, which demonstrates the possibility for functionalization at this secondary OH group.

Withanolide A (**1**) was evaluated with regard to its neuritogenic properties in the human SH-SY5Y neuroblastoma cell line according to a published method.[6a,17] Briefly, cells were grown on 24-well plates in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) in the presence of the corresponding compounds (1 μM, 0.1% DMSO) in a humidified atmosphere. After six days, cells were examined under a phase-contrast microscope. Those having at least one neurite with a length of more than 50 μm were counted as positives. In control experiments, cells were treated with vehicle (DMSO 0.1%, negative blank) and all-*trans* retinoic acid[18] (1 μM, positive blank). Pictures were taken from six random areas of the wells and evaluated for neurite positive cells. All assays were conducted in triplicate, and at least 300 cells were counted in each experiment. Error bars denote the standard error of the mean (SEM). To obtain more accurate results for withanolide A, more than 1500 cells were evaluated ($n = 18$–21). Interestingly, when we used conditions published by Tohda et al.,[6a,17] we were able to reproduce these results (DMSO vehicle 12% differentiated cells vs. withanolide A 22%, data not shown).

We then examined the same compounds on collagen-coated 24-well plates to support adhesion of the cells under otherwise identical conditions. The vehicle control experiments (0.1% DMSO) displayed a strikingly different phenotype compared to cells treated with withanolide A or retinoic acid (Figure 2). In the former case, we almost exclusively observed large cell aggregate formation with only few isolated

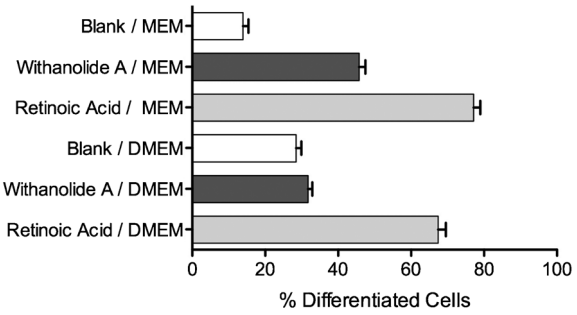



***Figure 2.*** Neurite outgrowth induced by withanolide A (**1**) and negative (DMSO) and positive (retinoic acid) controls in human SH-SY5Y cells in minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM). Error bars denote SEM. Representative micrographs are given in the Supporting Information. For MEM, the phenotypes induced by natural and synthetic withanolide A are in full agreement.

cells. Cells treated with withanolide A formed aggregates to a lesser extent, with many viable isolated cells that were often found to be differentiated. Both synthetic and isolated material displayed phenotypes that are in full agreement. Finally, treatment with retinoic acid completely suppressed aggregate formation and led to fully differentiated phenotypes.

We then incubated the cells in Dulbecco's modified Eagle's medium (DMEM, 10% FBS, antibiotics; higher nutrient concentration in the medium) in collagen-coated wells with the compounds and controls. In these cases, the large difference obtained for negative control experiments in MEM compared to withanolide A (14% vs. 46%) was reduced to a nonsignificant difference (28% vs. 32%). Also in rat cortical neurons, a reassessment of the neuritogenic activity has been proposed recently.[7] In the presence of DMEM, isolated non-differentiated cells appear to be more viable, thus leveling the observed ratios. These experiments therefore suggest that the neuritogenic phenotype observed in SY5Y cells appears to be conditional related to medium (MEM vs. DMEM) or coating of the wells. Further studies should thus address the question of whether withanolide A exerts a genuine neuritogenic effect.

Concerning the mechanism of action of withanolide A (**1**), a recent study by Chan and co-workers[7] reported that this steroid lactone is able to modulate several secretase targets relevant to neurodegeneration. Furthermore, this study reported the docking of withanolide A into beta-secretase 1 (BACE1) suggesting enzyme inhibition by binding.[7] We have evaluated the binding of withanolide A (**1**) against several proteases of potential relevance to neurodegenerative diseases (Table 1); however, in these assays withanolide A and derivatives **5** and **11** were found to be inactive up to a concentration of 100 μM. Only very weak activity was found against plasmepsin I (compound **5**) and plasmepsin I, II, and IV (compound **11**). While it remains feasible that withanolide A is able to modulate the expression of BACE1, as demonstrated by Chan and co-workers,[7] direct interaction of **1** with BACE1 as suggested[7] appears very unlikely based on the values reported herein.

***Table 1:*** Determined $IC_{50}$ values [μM] of compounds **1**, **5**, and **11** against targets potentially relevant to neurodegenerative diseases (BACE1 and cathepsin) and control enzymes (plasmepsin).

| | BACE1 | Cath D[a] | Cath E[a] | PM I[b] | PM II[b] | PM IV[b] |
|---|---|---|---|---|---|---|
| **1** | >100 | >100 | >100 | >100 | >100 | >100 |
| **5** | >100 | >100 | >100 | 67±4 | >100 | >100 |
| **11** | >100 | >100 | >100 | 46±14 | 52±5 | 31±7 |

[a] cath = human cathepsin. [b] PM = plasmepsin.

In conclusion, we have reported the first successful preparation of the pharmacologically important steroid lactone withanolide A (**1**) from a simple precursor and have evaluated its neuritogenic properties and mechanism of action related to secretase targets. Notable features of this synthesis include: 1) a highly diastereoselective singlet-oxygen-mediated photooxygenative olefin migration, 2) a strategy that minimizes the use of protecting groups and exploits the inherent reactivity pattern in the endgame, and 3) a Wharton transposition for the establishment of the A ring. In addition, biological studies of neurite outgrowth in human SH-SY5Y cells demonstrated the dependence of neuritogenic properties on parameters such as medium and surface coating. Concerning the mechanism of action, direct binding of withanolide A (**1**) to secretase targets as suggested earlier[7] appears unlikely, as shown by enzyme assays. These results on the synthesis, derivatization, and neuritogenic activity open the way for more detailed studies on the mechanism of action of withanolide A (**1**), which will be carried out in our laboratories.

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[19] CCDC 816947 (**5**), 816948 (**7**), 816949 (**10**) 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/data_request/cif.

  