Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2467–2469

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis, Chiral Chromatographic Separation, and Biological Activities of the Enantiomers of 10,10-Dimethylhuperzine A

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Received 17 July 2000; accepted 21 August 2000

Abstract—(±)-10,10-Dimethylhuperzine A (**2**, DMHA) has been synthesized, and its enantiomers have been separated using chiral HPLC. (−)-DMHA inhibits AChE with a K_i value approaching that of (−)-huperzine A, whereas (+)-DMHA shows no AChE inhibitory activity. On the other hand, both enantiomers are equally potent against glutamate-induced neurotoxicity when tested in neurons.

Alzheimer's disease (AD) is one of the major diseases affecting the elderly population throughout the world. The disease is a progressive neurodegenerative disorder that has been linked to the deposition of amyloid plaques and neurofibrillary tangles, as well as with reduced chemical neurotransmission in the brain.[1] One of the major neurochemical deficits associated with AD is a dysfunction in the cholinergic system, resulting from a deficiency in the neurotransmitter acetylcholine. This small molecule has been shown to play a fundamental role in memory and learning.[2] Several therapeutic strategies have been explored to enhance cholinergic neurotransmission in order to alleviate some of the symptoms of AD. These include inter alia the use of acetylcholinesterase (AChE) inhibitors, the administration of acetylcholine precursors, the investigation of acetylcholine releasers, as well as the identification of direct acetylcholine receptor agonists (both muscarinic and nicotinic).[3] Among these strategies, acetylcholinesterase inhibition has proven to be the most successful means to balance the cholinergic deficit and to stabilize the symptomatology.[4] Because of the partial success that has been achieved with the use of AChE inhibitors, considerable effort has been expended in identifying other more potent, selective, and safe AChE inhibitors.[5]

Huperzine A (HA) (**1**), a Lycopodium alkaloid, has been isolated from the clubmoss *Huperzia serrata* (Thunb.) Trev. = *Lycopodium serratum* Thunb., a Chinese traditional medicine, and has attracted considerable attention for its possible use in the treatment of AD.[6–8] This natural product has proven to be a very potent and selective reversible inhibitor of AChE with almost no action on butyrylcholinesterase. The superior inhibition properties of HA have been attributed to its very slow dissociation ($t_{0.5} = 35$ min) from the enzyme and its long duration of action. In double-blind, placebo-controlled studies conducted in China, HA has been found to dramatically improve the cognitive performance of individuals suffering from various forms of memory impairment, and it has been approved and clinically used as a palliative agent for AD in China.[8] Another interesting aspect of HA's pharmacology relates to its neuroprotective properties against glutamate-induced cell death.[9] Because of the tremendous promise this alkaloid holds for the treatment of AD, several groups have been engaged in intensive efforts to explore its structure–activity relationships.[6–8] We have reported previously the first total synthesis of (±)-HA, (−)-HA, numerous analogues,[7] and an X-ray structural analysis of HA in complex with *Torpedo* AChE.[10] To date, we have identified several analogues of HA that have better or comparable activity to that of the parent structure. In particular, the C-10 axial methyl analogue of (±)-HA was found to be 8-fold more potent than (±)-HA, the (−)-10-spirocyclopropyl analogue was nearly as active as (−)-HA, and (±)-DMHA (**2**) was

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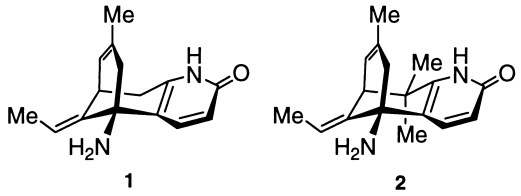
0960-894X/00/$ - see front matter
PII: S0960-894X(00)00494-7

found to be about equipotent to HA but with a slower off-rate from the enzyme.[11,12]



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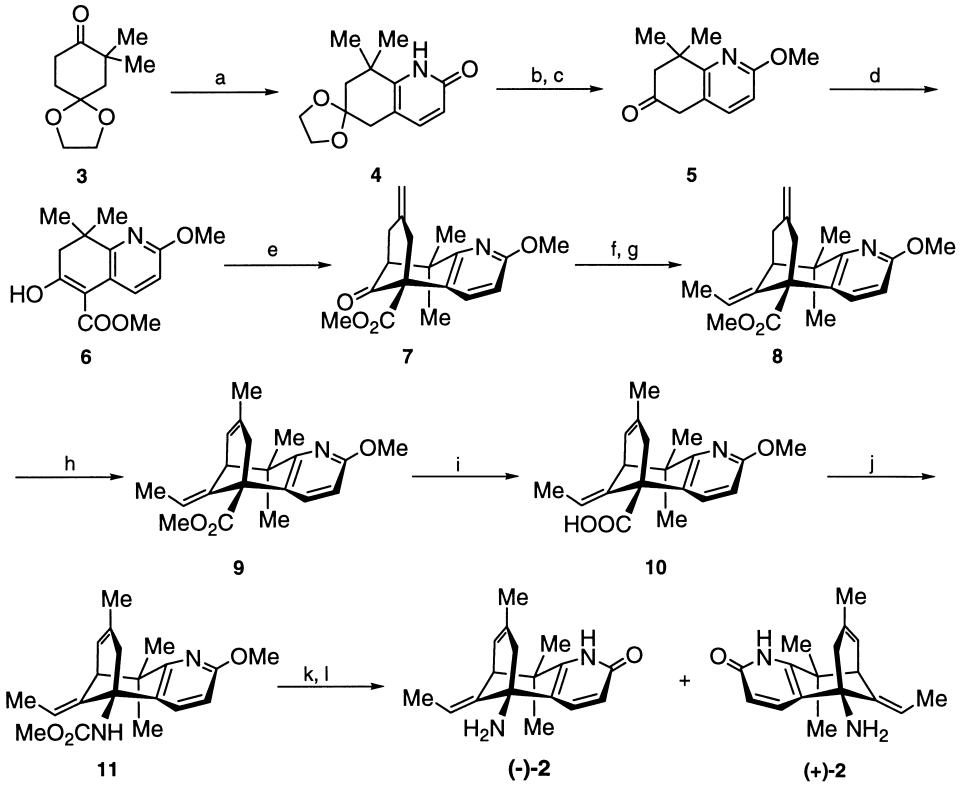
In continuation of our studies on these potent C-10 methyl substituted HA analogues, we chose to examine the activity of the (−)- and (+)-enantiomers of DMHA (**2**) in AChE inhibition and in a glutamate toxicity model.

Chemical Synthesis

The synthesis of *rac*-**2** was performed according to the literature procedure starting from ketone **3** (Scheme 1).[13] In brief, ketone **3** was treated with ethyl propiolate and ammonia in methanol at 100 °C in a pressure reactor for 8 h to give the fused ring pyridone **4** in 65% yield. After conversion of the pyridone to methoxypyridine by *O*-methylation employing Ag_2CO_3/MeI, the ketal was deprotected with acetone/HCl under reflux to obtain compound **5**. Reaction of ketone **5** with 2.2 equiv of NaH and 4.0 equiv of dimethyl carbonate yielded the β-ketoester **6** in good yield. The β-ketoester **6** was treated with 2-methylene-1,3-propanediol diacetate, DBU, and a catalytic amount of $Pd(OAc)_2/PPh_3$ to provide the annulation product **7** in 85% yield. The standard Wittig olefination of tricyclic compound **7** with $Ph_3P^+Et\ Br^-$ and *n*-BuLi gave the *Z*-olefin (structure not shown, *Z:E* = 8:2) as the major isomer which was isomerized to the *E*-isomer **8** with thiophenol and AIBN (*Z:E* = 1.5:8.5). The second isomerization (exocyclic to endocyclic double bond) was accomplished by treating **8** with trifluoromethanesulfonic acid in 1,4-dioxane at 90 °C. Saponification of the resulting ester **9** with 6 N NaOH in THF:methanol (1:2) at reflux afforded the pure *E*-acid **10** (the *Z*-isomer failed to undergo hydrolysis). Next, modified Curtius reaction of acid **10** with diphenyl azidophosphate and Et_3N followed by methanolysis of the resulting isocyanate provided urethane **11** in 85% yield. Finally, double deprotection of **11** with iodotrimethylsilane in refluxing chloroform gave (±)-DMHA (**2**).

To separate racemic DMHA into its individual enantiomers, HPLC was performed using a chiral column containing amylose tris(3,5-dimethylphenyl carbamate) coated on a 10 μm silica gel substrate and hexanes/ethanol/diethylamine (90/10/0.05) as the eluent. This was conducted on a semi-preparative scale (2.1 mg of racemate per injection) on a 25×1.0 cm column.[14] The optical rotation of (−)-**2** (98.0% ee) was found to be $[\alpha]_D$ −64.2° (*c* 0.605, $CHCl_3$) and that of (+)-**2** (98.7% ee) was $[\alpha]_D$ +64.2° (*c* 0.60, $CHCl_3$).[15]



Scheme 1. Reagents and conditions: (a) ethyl propiolate, NH_3, MeOH, 100 °C, 8 h, 65%; (b) Ag_2CO_3, MeI, $CHCl_3$, reflux, 2.5 h, 95%; (c) acetone/3 N HCl (1:1), reflux, 3 h, 90%; (d) 2.2 equiv NaH, 4.0 equiv $(MeO)_2CO$, THF, reflux, 3.5 h, 87%; (e) $(CH_3CO_2CH_2)_2C{=}CH_2$, $Pd(OAc)_2/PPh_3$ (1:4.1), DBU, 1,4-dioxane, rt, 0.5 h, then reflux, 3.5 h, 85%; (f) $CH_3CH_2P^+Ph_3Br^-$, *n*-BuLi, THF, rt, 15 h (*Z:E* = 8:2); (g) PhSH, AIBN, toluene, 95 °C, 24 h (*Z:E* = 1.5:8.5), 60% over two steps; (h) CF_3SO_3H, 1,4-dioxane, 90 °C, 13 h, 98%; (i) 6 N NaOH:MeOH:THF (1:2:1) reflux, 85%; (j) $(PhO)_2P(O)N_3$, Et_3N, toluene, reflux, 3.5 h, then MeOH, reflux, 10 h, 85%; (k) TMSI, $CHCl_3$, reflux, 8 h, then MeOH, reflux, 24 h, 95%; (l) chiral HPLC separation (see text).

Table 1. AChE inhibition parameters for huperzine A and the 10,10-dimethylhuperzine A analogues

Inhibitor	K_i (nM)
(−)-HA	5.9
(±)-DMHA	16.7
(−)-DMHA	7.7
(+)-DMHA	30.9×10^3

Table 2. Effect of pretreatment with the enantiomers of DMHA on AChE activity and the survival of embryonic forebrain neurons when exposed to glutamate

	Neuronal survival	AChE activity
Treatment	% of control	% of control
None	100.2±1.5	99.9±1.1
Glutamate	43.7±2.9	78.3±4.1
Glu + (−)-DMHA	71.7±3.8	2.3±0.5
Glu + (+)-DMHA	71.3±5.0	75.0±1.7

Biological Activity

The AChE inhibitory activity of (−)-**2** and (+)-**2** was assayed using AChE isolated from fetal bovine serum[16] as described previously,[12] using acetylthiocholine as the substrate.[17] The K_i values obtained using the steady state method are reported in Table 1. Remarkably, optically pure (−)-DMHA is comparable in activity to natural (−)-HA while (+)-DMHA (**2**) is basically inactive. Thus, the enzyme shows exquisite specificity in discriminating between the two enantiomeric forms of DMHA.

To determine the effect of the DMHA enantiomers on cell survival in a glutamate model of neurotoxicity, cell cultures were prepared from rat embryonic forebrain. The cultured neuronal cells were pretreated with either (−)-DMHA or (+)-DMHA (500 nM) and then challenged with glutamate (75 μM). The cell viability was measured after 24 h using the MTT dye, and acetylcholinesterase activity was measured in the same culture using a modified Ellman's assay (Table 2).[18] The glutamate-alone treatment resulted in approximately 57% cell death while AChE activity was reduced by approximately 22%. Pretreatment of the cell cultures with either enantiomer of DMHA results in an equivalent level of neuroprotection amounting to ~71% survival with either enantiomer. The AChE activity was almost completely reduced with (−)-DMHA. Thus, as (+)-DMHA lacks any significant AChE inhibitory activity, it is clear that the neuroprotective properties of these compounds must be a consequence of an action at another site. Recent work suggests that the neuroprotective properties of huperzine and its analogues may result from blockade of calcium mobilization through the NMDA receptor.[8] This idea is being further tested through an examination of the ability of the enantiomeric forms of DMHA to alter MK-801 binding at the NMDA receptor.

Acknowledgements

We are indebted to the Department of Defense (DMAD17-93-V-3018) for support of these studies.

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14. The chiral HPLC was performed on a Shimadzu LC 8A HPLC system with a CHIRALPAK® AD™ column (Daicel Chemical Industries, Ltd) at a flow rate of 4.0 mL/min at room temperature and UV detection at 230 nm. The sample for injection was prepared by first dissolving (±)-DMHA (**2**, 21 mg) in ethanol (1 mL) and then diluting with the mobile phase (2 mL). Retention time (t_R) = 10.3 min for (+)-DMHA and 11.9 min for (−)-DMHA.
15. Optical purities were measured by resubjecting the pure enantiomers to the same chiral HPLC method and are based upon the computerized integration of peak areas.
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