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SYNTHESIS AND ACTIVITY STUDIES OF N-[ω-N'-(ADAMANT-1'-YL)AMINOALKYL]-2-(4'-DIMETHYLAMINOPHENYL)ACETAMIDES: IN THE SEARCH OF SELECTIVE INHIBITORS FOR THE DIFFERENT MOLECULAR FORMS OF ACETYLCHOLINESTERASE

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Abstract: A series of N-[ω-N'-(adamant-1'-yl)aminoalkyl]-2-(4'-dimethylaminophenyl)acetamides were synthesized and tested as acetylcholinesterase inhibitors. A significant selectivity toward acetylcholinesterases from various natural sources, mainly differing in their quaternary structure and solubility, was pointed out. The interest of this kind of molecules as potential therapeutic agents for Alzheimer's disease is discussed.
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Alzheimer's disease (AD) is characterized by a strong decrease in the functionality of the central cholinergic system, resulting in several cognitive disfunctions[1]. Clinical studies have demonstrated that the inhibition of acetylcholinesterase (AChE) in the brain can produce a significant memory enhancement in patients affected by AD. Two inhibitors, tacrine and E2020, have been approved so far for the therapeutic use, while several others are currently under clinical experimentation[2]. Great progresses have been made since the discovery of physostigmine, a potent but short lasting and toxic inhibitor[3]. The duration of the inhibition has been enhanced, and the side effects, due to the action on the peripheral nervous system, have been strongly reduced by increasing the penetration of the blood-brain barrier with consequent reduction of the peripheral concentration of inhibitor[2]. Nevertheless, along the way to the discovery of the ideal AChE inhibitor, another possibility can be further explored. Since AChE is present in the organism in several forms, mainly differing in their quaternary structures[4], it would be interesting to search for a selective inhibitor of one or some of them. A selective inhibitor of the cerebral enzyme could solve the problem of the side effects due to the peripheral action. Moreover, as shown by previous studies, in the AD brain, the proportion among the different forms of the enzyme is altered as compared to the normal brain, and the preferential inhibition of some of them could restore the initial equilibrium[5]. Finally, from the structure of a selective inhibitor, important information could be deduced on the influence of the quaternary structure on the binding properties of the enzyme.

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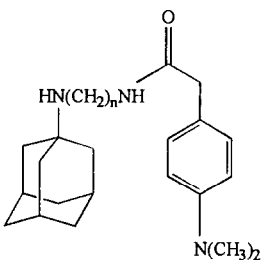
The multiple molecular forms of AChE can be distinguished based on their shapes in collagen-tailed asymmetric forms (A_n) and globular forms (G_n). The globular forms can be subdivided on the basis of their solubility into two main classes: detergent-soluble forms (G_n^D) and aqueous or low salt-soluble forms (G_n^A). The asymmetric forms are all hydrophilic and homologously high salt-soluble. In mammalian brain, the prevailing form is the G_4 hydrophobic-tailed, which is formed by a tetramer of catalytic subunits anchored to the synaptic membrane by a smaller structural hydrophobic subunit. The monomeric G_1 form is present in minor amount, while the others are present in almost negligible amount. In the brain of AD patients there is a strong reduction of the activity of the G_4 form, while there is only a little decrease of the activity of the G_1 form as compared to the normal brain[5]. The positive implications of a preferential inhibition of the latter have been underlined in a study, conducted on human brain tissue, on the carbamic inhibitor SDZ-ENA 713 (Sandoz), which is presently in advanced clinical trial[6].

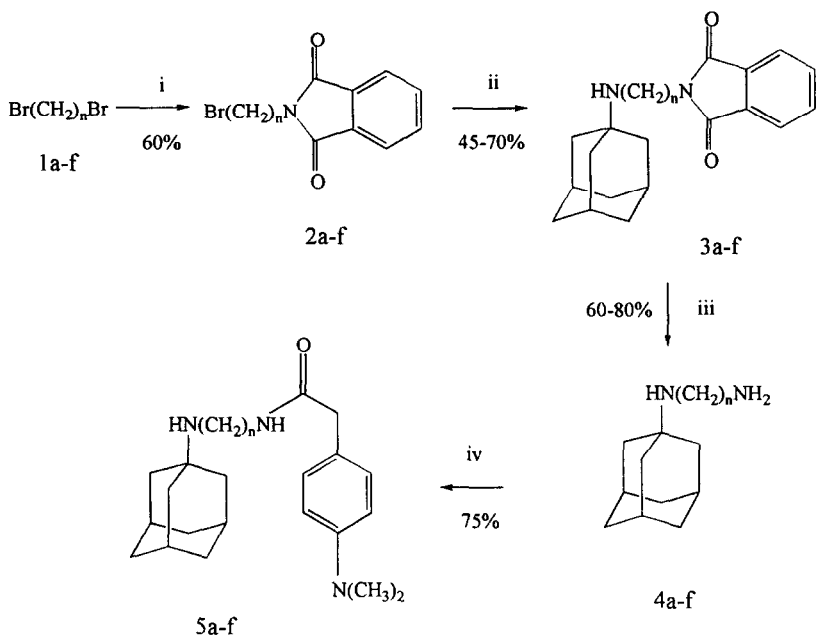
In order to investigate the possibility of finding selective inhibitors for the various forms of the enzyme, a new homologue series of compounds has been synthesized, and checked for their inhibitory properties of various AChEs. On the basis of the known structural properties of the enzyme[7], the basic structure of the new inhibitors has been designed as follows:



(7 < n < 12)

The active site of AChE is constituted by a catalytic triad and a so-called "main anionic site", both located at the bottom of a deep narrow gorge. At the entrance of the gorge, there is a regulatory site called "peripheral anionic site". The crystal structure of AChE from Torpedo californica has revealed that tryptophan residues play a central role in both secondary sites[7,8]. In the above structure, the aromatic amino group, the amide function and the aliphatic amine have been introduced to interact with the main anionic site, the catalytic triad and the peripheral anionic site of the enzyme, which are common to all forms. The adamantyl group should be the probe to locate the differences in the binding properties of such forms, resulting from the different quaternary structures: as a result of its size and hydrophobicity it should be able to discriminate among the more and the less hindered active site gorges, as well as to interact with the hydrophobic sites specific of some forms of the enzyme.

SCHEME 1



Reagents and conditions: i) Potassium phthalimide, DMF, reflux, 2 h; ii) 1-adamantylamine, N-ethyl-N',N''-diisopropylamine, CH_3CN, 60°C, 4 h; iii) NH_2NH_2, MeOH, reflux, 4 h; iv) p-dimethylaminophenylacetic acid, N-methylmorpholine, isobutyl chloroformate, THF, -10°C, 1.5 h, then 0°C, 1.5 h.

The synthesis of the inhibitors was accomplished according to scheme 1. The bromoalkylphthalimides **2a-f** (n=7 to 12) were synthesized by reacting potassium phthalimide with three equivalents of the alkyldibromides **1a-f** in refluxing DMF for 2 h. The reaction products were purified by column chromatography on silica gel, eluted with a mixture chloroform/ n-hexane 7:3 or 8:2. Compounds **2a-f** were obtained in yields around 60 %. These compounds were then used to alkylate adamantylamine; 3 equivalents of amine were used to avoid the formation of the bridged diamine. The reaction was performed in acetonitrile at 60°C for 4 h in the presence of 1 equivalent of diisopropylethylamine. The reaction mixture was then chromatographed on a column of silica gel, first eluted with chloroform, to remove the fraction of not reacted n-bromoalkylphthalimide, and then with chloroform/ methanol 95:5 or 9:1. The products **3a-f** were isolated with yields ranging from 45 to 70 %. In the subsequent step, the phthalimido group was removed by hydrazinolysis, performed in refluxing methanol for 4 h. The standard procedure was followed to isolate the products **4a-f**, obtained in yields ranging from 60 to 80 %. The final products were obtained by reacting compounds **4a-f** with p-dimethylaminophenylacetic acid, previously activated with isobutyl chloroformate. The activation was performed by adding 1 equivalent of N-methylmorpholine to a solution of p-dimethylaminophenylacetic acid in THF at -10°C followed by 1 equivalent of isobutyl chloroformate. After addition of the diamines **4a-f** to the activated intermediates, the coupling

reactions were carried out for 1.5 h at -10°C followed by 1.5 h at 0°C. The final products **5a-f** were purified by column chromatography on silica gel, eluted with ethyl acetate/ methanol 7:3. The yields of this last step were around 75 %. The compounds were characterized by ^{1}H NMR and MS.

The inhibitory properties of compounds **5a-f** have been first evaluated by studying their kinetics of inhibition on AChE from Torpedo californica. This enzyme has been chosen in order to rationalize the results on the basis of its known crystallographic structure. Esterase activity has been measured according to Ellman's method[9]. The inhibition is in all cases time-independent, and all the inhibitors display a mixed-linear kinetics of inhibition. The values of K_I and K_I', which can be related to the contributions of the competitive and of the non competitive components of the inhibition respectively, are reported in Table 1.

Table 1. Inhibition constants of Torpedo californica AChE by **5a-f**

Inhibitor	n (CH_2)	K_I (μM)	K_I' (μM)
5a	7	7 ± 2	13 ± 2
5b	8	5.5 ± 0.4	10 ± 3
5c	9	3.4 ± 0.8	7.0 ± 0.7
5d	10	2.5 ± 0.6	5.3 ± 0.5
5e	11	2.1 ± 0.3	5.2 ± 0.9
5f	12	1.8 ± 0.4	4.1 ± 0.7

On the basis of these results, the following observations can be made:

1) the values of K_I' are comparable to those of K_I. This confirms the importance for this kind of inhibitors of the interactions with sites different from the catalytic site, which are responsible for the non competitive component of the inhibition;

2) the inhibitory power increases with the length of the aliphatic chain. The increase is significant from the C_7 to the C_{10} derivative. According to the hypothesis that these inhibitors can bridge the main anionic site and the peripheral anionic site of the enzyme, this trend agrees with the models and with previous results that indicated C_{10} as the ideal length for this type of interaction[10]. The slight increase of the inhibitory power from the C_{10} to the C_{12} derivative might indicate the occurrence of hydrophobic interactions between the adamantyl group and any hydrophobic site located near the entrance of the catalytic pocket.

In order to evaluate the potential selectivity of these compounds toward the various forms of AChE, the *in vitro* inhibitory power (IC_{50}) has been measured on various AChEs available from natural sources, differing in their degree of association and/or in their chemico-physical state. Since there is a high sequence homology among the AChEs from different sources, especially in the region of the active site[4], these enzymes can be

regarded as models of the different molecular forms of the human enzyme, of which only one is commercially available.

The inhibitors have been tested on five AChEs: a water-soluble dimer from Torpedo californica, a water-soluble tetramer from electric eel, a mixture of membrane-bound oligomers (from 2 to 8 subunits) from bovine erythrocytes, a membrane-bound tetramer from bovine brain and a membrane-bound tetramer from human erythrocytes. The measurements have been performed on the inhibitors with an aliphatic chain length of 8, 10 and 12 carbon atoms. As a reference, physostigmine (Phy) and E2020 have been tested on the same enzymes.

Table 2. AChE inhibition, IC_{50} μM (±10%)

Source	Molecular form	5b	5d	5f	Phy	E2020
Torpedo californica	Dimer, water soluble	10	5.3	3.3	0.046	0.012
Electric eel	Tetramer, water soluble	28	14	8.9	0.046	0.017
Bovine erythrocytes	Oligomers(2-8), membrane bound	>100	95	19	0.123	0.008
Bovine brain	Tetramer, membrane bound	>100	77	20	0.146	0.006
Human erythrocytes	Tetramer, membrane bound	>100	>100	>100	0.047	0.008

As shown in Table 2, physostigmine and E2020, which don't have a relevant steric hindrance and bind specifically inside the catalytic pocket of the enzyme[11,12], display scarce selectivity towards the different AChEs. This agrees with the statement that the structure of the active site gorge is widely conserved. Concerning the new inhibitors, the following observations can be made:

a) the same relationship between the chain length and the inhibitory power is observed on all the examined AChEs. This is a further confirmation that the relative positions of the main sites of interactions are conserved;

b) there is a relevant difference in the inhibitory power displayed by each compound toward the different AChEs. In particular compound **5f** displays a difference of two orders of magnitude in the inhibitory power on the Torpedo californica dimer as compared to that on the human erythrocytes tetramer. Such result is even more remarkable if the very high sequence homology between the two enzymes[13] is considered. This confirms that this kind of inhibitors are suitable to discriminate among the various forms of the enzyme, probably on the basis of interactions other from those involving the catalytic pocket, which is nearly the same in all forms.

An attempt to rationalize such differences can be made considering that the examined inhibitors are more active on the soluble than on the membrane-bound AChEs, and that among the former the dimer is more strongly inhibited than the tetramer. According to the hypothesis that the differences in the binding properties can be related to the quaternary rather than to the primary structure of the enzyme, this means that the more complex is the type of association, the more disfavourable is the interaction with this kind of inhibitors. This can be

explained supposing that the subunits of the enzyme have the general tendency to associate to each other and to the membrane in a way that partially hinders the access to the catalytic pocket. In view of the high sequence homology between human and Torpedo californica AChE, this would lead to the hypothesis that these compounds, and especially **5f**, could discriminate between the monomeric and the tetrameric form of the human enzyme by preferentially inhibiting the first one. A remarkable selectivity between the G_1 and the G_4 forms could be the ideal tool for restoring the right proportion between their activities in the AD brain. Experiments on brain tissue will be needed to confirm the validity of this hypothesis.

In conclusion the above results, besides supporting the possibility of creating a selective inhibitor, provide the basis for further investigations on the influence of the quaternary structure on the binding properties of AChE. They suggest that the accessibility to the catalytic pocket may be limited by the association of the subunits.

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