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Allobetulin derived *seco*-oleananedicarboxylates act as inhibitors of acetylcholinesterase.

Lucie Heller, Stefan Schwarz, Anja Obernauer and René Csuk*

Martin-Luther-Universität Halle-Wittenberg, Bereich Organische Chemie, Kurt-Mothes-Str. 2, D-06120 Halle (Saale), Germany; e-mail: rene.csuk@chemie.uni-halle.de

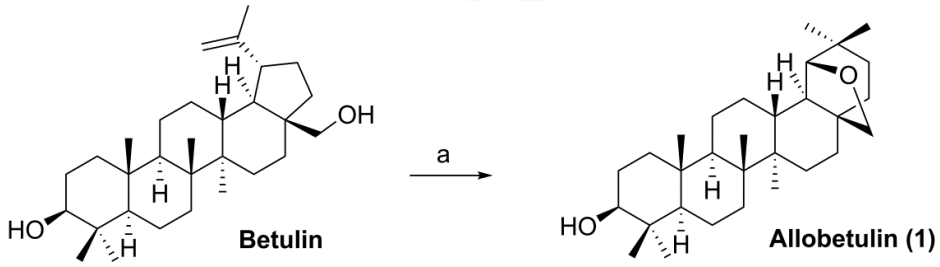
Abstract – Ring opening of allobetulone gave either *seco*-acid **8** or di-acid **4**. These acids were converted into esters that were screened by Ellman's assay. A dibutenylester of low cytotoxicity (NIH 3T3 murine embryonic fibroblasts) was shown to be a good mixed-type inhibitor (K_i = 3.39, K_i' = 2.26 μM) for acetylcholinesterase.

Worldwide approximately 35 million individuals suffer from some kind of dementia; 20 million of them are affected by Alzheimer's disease (AD), and within the next 30 years this number will double.[1-4] AD is a form of pre-senile dementia mainly diagnosed in people whose ages are over 65, but is not exclusively an aging-related disease.[5,6] A significant number of all AD cases is known as the early onset familial AD (fAD),[5] and their symptoms appear in young age. But the vast majority of AD cases, however, are classified as sporadic AD (sAD) being triggered by genetic as well as non-genetic factors, and its prevalence increases with age.[7-9] Regardless of their age, all of these AD patients lose their short-term memory, there is a progressive decline in attention, languages and spatial reasoning, and finally all of these patients will withdraw from social life, and they will be no longer able to execute daily life activities at all.[1-5]

The impairment on the cholinergic function seems of critical importance in AD ("cholinergic hypothesis"),[1] and the levels of the neurotransmitter acetylcholine (ACh) are decreased in AD brains. In addition, during the development of AD an increased activity of butyrylcholinesterase (BChE) was found in the hippocampus and the temporal cortex.[1]

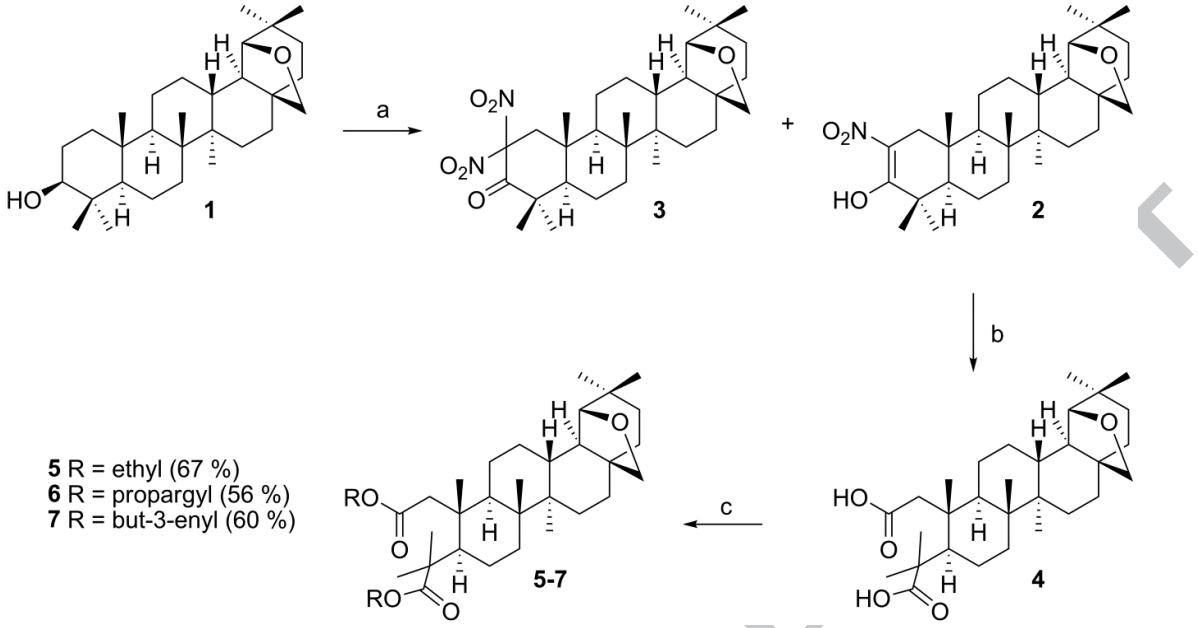
Several cholinesterase inhibitors are commercially available to alleviate the symptoms of AD, and drugs such as galantamine, tacrine, rivastigmine and donepezil and derivatives thereof are successful in slowing down the process of cognitive impairment.[1]

Recently, several triterpenes came into the focus of scientific interest concerning the treatment of AD related symptoms. [10-18] In search of novel inhibitors of AChE we used Ellman's assay to screen our library of triterpenoids, containing > 1400 derivatives of triterpenoic acids. While many compounds showed low or no activity at all, several triterpenoids [10, 19] especially derivatives derived from allobetulin (**1**, Scheme 1) [20] gave some promising results; **1** can be regarded as a "re-arranged" betulin derivative.[21] The hits from this primary screening, however, possessed only low solubility. Probably due to this low solubility usually observed with derivatives of allobetulin, the number of biological investigations of allobetulin remained small over the years.[22-31] Hence, we decided to search for allobetulin derivatives of improved solubility, low cytotoxicity and significant ability to inhibit AChE.



Scheme 1. Structure of betulin and synthesis of allobetulin (**1**): a) *p*-TSA, $CHCl_3$, reflux 1 hour, according to ref. 32, 75 %.

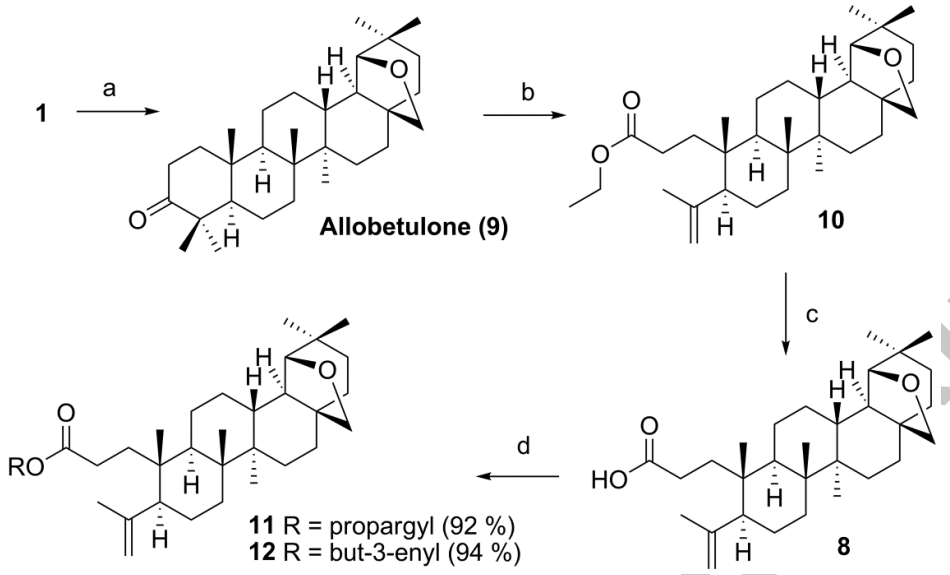
Parent allobetulin (**1**, Scheme 1) can be obtained in 75 % isolated yield from betulin by its reaction with *p*-TSA.[32] From the reaction of **1** with acetic acid and nitric acid [33] a mixture of mononitro-**2** and dinitro-**3** was obtained (Scheme 2); this mixture was easily separated by chromatography. Compound **2** was characterized in its ^{13}C NMR spectrum by two signals at δ = 123.0 and 176.3 ppm that were assigned to enolic carbons C-2 and C-3, respectively. In its IR spectrum the signals at ν = 1513 and 1376 cm^{-1} were attributed to the NO_2 valence vibrations. In the dinitro compound **3**, the carbonyl group at C-3 was detected at δ = 197.4 ppm, while the signal of the dinitro substituted carbon C-2 was found at δ = 119.4 ppm.



Scheme 2. Synthesis of nitro-compounds **2** and **3**, *seco*-diacid **4** and esters **5**-**7**: a) HOAc, HNO_3, 25 °C, 1 h, 70 % (of **2**) and 8 % (of **3**); b) MeOH, H_2O_2, K_2CO_3, 25 °C, 5 days, acidic workup, 78 %; c) K_2CO_3, DMF, R-X, 25 °C, 12 h.

Reaction of **2** with potassium carbonate and hydrogen peroxide in methanol for five days gave *seco*-diacid **4** [33, 34] in an isolated yield of 78 %. This compound is highly insoluble in most organic solvents. Its esterification with alkyl halides in the presence of finely powdered potassium carbonate yielded the esters **5**-**7** showing improved solubility.

For comparison, the mono-acid **8** and the mono esters **10-12** were prepared starting from **1**. Thus, Jones oxidation of **1** furnished allobetulone (**9**, Scheme 3) [34] whose treatment with oxone ® and conc. sulfuric acid in ethanol furnished ring opened *seco*-ethyl ester **10**. [35] The formation of this *seco* compound can be explained by a Bayer-Villiger re-arrangement of **9** followed by a transesterification of the transient lactone and an elimination reaction of a tertiary alcohol.



Scheme 3. Synthesis of compounds **8**-**12**: a) CrO_3, H_2SO_4, acetone, 81 %; b) oxone®, EtOH, H_2SO_4, 25 °C, 7 days, 65 %; c) MeOH, KOH, 25 °C, 7 d, 87 %; d) R-X, K_2CO_3, DMF, 25 °C, 12 h.

Reaction of this ester **10** with methanolic potassium hydroxide for one week gave acid **8**, whose esterification with propargyl bromide or 4-bromo-1-butene in DMF as described above gave the esters **11** and **12**, respectively.

Allobetulin, monoacid **8**, diacid **4** and esters **5**-**7** and **10**-**12** were subjected to Ellman's assay [36, 37] to determine their inhibitory activity for the enzymes AChE (from *Electrophorus electricus*) and BChE (from equine serum). Pre-screening showed all of these compounds (as well as of other monoalkyl- and dialkyl-esters that were easily accessed by esterification of **4** and **8**, respectively) as weak inhibitors of AChE and BChE, hence we refrained from a more extensive screening with these enzymes. The ethyl esters **5** and **10** were weak inhibitors of AChE; the solubility of **12** in aqueous buffers was low. However, these experiments showed **8** as a moderate competitive inhibitor for AChE with a K_i = 31.78 ± 4.28 µM (IC_{50} = 103.5 ± 9.7 µM) , while the di-propargyl ester **6** was a mixed-type inhibitor for this enzyme with K_i = 10.45 ± 3.71 µM (competitive part) and K_i' = 4.63 ± 0.63 µM (uncompetitive part) with IC_{50} = 6.3 ± 0.2 µM. Better results were obtained for the mono-propargyl ester **11** (K_i = 3.33 ± 1.15 *m*M, K_i' = 2.48 ± 0.06 µM; IC_{50} = 6.7 ± 0.1 µM) and the di-butenyl ester **7** showing inhibition constants in the single digit range of K_i = 3.39 ± 1.13 µM and K_i' = 2.26 ± 0.36 µM (IC_{50} = 8.7 ± 0.2 µM). For comparison, for standard compound serine IC_{50} values of. 0.015 µM – 0.11 µM (for AChE) and 0.016 µM – 0.218 µM (for BChE) have been reported.[38-40]

A low cytotoxicity seems mandatory for compounds to treat AD. Thus, EC_{50} values were measured using the photometric sulforhodamine B assay [31-44] employing murine embryonic fibroblasts (NIH 3T3).

The results from the SRB assay gave an EC_{50} for **8** of > 30 µM but also the best inhibitors of AChE (mono-propargyl **11** and di-butenyl **7**) of rather low cytotoxicity (> 30 µM, cut-off of the assay), while the di-propargyl ester **6** (EC_{50} = 7.0 ± 2.5 µM) seems not suited due to high cytotoxicity.

In conclusion, we report the synthesis of a small series of allobetulin derived *seco* compounds. The results from Ellman's-assays showed these compounds as good inhibitors of acetylcholinesterase. Their low cytotoxicity qualifies some of these compounds (and derivatives thereof) for further studies in the field of AD research inasmuch as the development of AChE/BChE selective inhibitors might reduce udesired side effects as associated with dual inhibitors and slow down AD progress especially at the beginning of the disease. In addition, the administration of inhibitors with different AChE/BChE selectivity might be useful as AD progresses.[45]

Acknowledgments

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Supplementary data

Supporting information is available (experimental procedures and full analytical data of all compounds). This material is available free of charge via the internet at http://sciencedirect.com.

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Graphical Abstrac

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