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Research paper

Converting maslinic acid into an effective inhibitor of acylcholinesterases

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ABSTRACT

During the last decade, maslinic acid has been evaluated for many biological properties, *e.g.* as an anti-tumor or an anti-viral agent but also as a nutraceutical. The potential of maslinic acid and related derivatives to act as inhibitors of acetyl- or butyryl-cholinesterase was examined in this communication in more detail. Cholinesterases do still represent an interesting group of target enzymes with respect to the investigation and treatment of the Alzheimer's disease and other dementia illnesses as well. Although other triterpenoic acids have successfully been tested for their ability to act as inhibitors of cholinesterases, up to now maslinic acid has not been part of such studies.

For this reason, three series of maslinic acid derivatives possessing modifications at different centers were synthesized and subjected to Ellman's assay to determine their inhibitory strength and type of inhibitory action. While parent compound maslinic acid was no inhibitor in these assays, some of the compounds exhibited an inhibition of acetylcholinesterase in the single-digit micro-molar range. Two compounds were identified as inhibitors of butyrylcholinesterase showing inhibition constants comparable to those of galantamine, a drug often used in the treatment of Alzheimer's disease. Furthermore, additional selectivity as well as cytotoxicity studies were performed underlining the potential of several derivatives and qualifying them for further investigations. Docking studies revealed that the different kinetic behavior within the same compound series may be explained by the ability of the compounds to enter the active site gorge of AChE.

1. Introduction

Many people living in the western hemisphere, and especially those of an advanced age, are afraid of spending their last years suffering from serious diseases, *e.g.* from cancer or a stroke. Dementia illnesses – like the Alzheimer disease (AD) – considerably contribute to uncertainty among the population due to their impact on cognitive abilities. Worldwide approximately 35 million individuals, *i.e.* one in 200, currently suffer from some kind of dementia; according to some estimates within the next 30 years this number might double [1,2]. 20 million of these persons are affected by AD, and the prevalence of AD increases with age starting from 10% at the age of 65 to nearly 50% at 85 years [1]. Therefore, there is a scientific and economic demand for further investigations for a better understanding as well as for finding effective treatments.

Although AD is not completely understood, several hypotheses exist and are foundations or at least starting points of current therapies. One of these theories ("amyloid hypothesis") deals with the neurotoxic effect of a β-amyloid being formed by the action of α-, β- and γ-secretases [3,4]. Although this is one of the most prominent theories, there are some deficits: For instance, approximately 30 per cent of healthy, middle-aged people possess equal amounts of β-amyloid plaques being usually found in AD brains [5,6]. Most of the therapies aiming to decrease the concentration of the plaques, however, did not result in permanent increase of cognitive abilities or their at least their restauration [7–10]. Other attempts to find possible treatments focus on the investigation of inflammatory processes, mitochondrial disorders or the τ-protein [11–13].

Impairment in the cholinergic function, however, is of critical importance in AD ("cholinergic hypothesis") [1,14]. Thus, our

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present study focusses on the neurotransmitter acetylcholine (ACh), whose concentration is reduced during AD leading to typical symptoms like amnesia or behavioral disorder [14–17]. The hydrolytic enzymes acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C. 3.1.1.8) are responsible for the hydrolysis of ACh, thus, controlling the concentration of this neurotransmitter in different tissues of an organism. Therein, BChE serves as a coregulator of the cholinergic transmission, and although it is mainly present in other parts of the body, BChE is able to compensate a reduced AChE activity in the brain [1,18,19]. Furthermore, the AChE/BChE ratio in the brain alters from 0.2 in normal brain to 11 during AD [14,20,21]. For this reason, both enzymes represent interesting targets for the development of AD therapies or tools for a deeper insight into this disease.

The only treatments with clinical evidence [14] to AD patients are the cholinesterase inhibitors galantamine, donepezil and rivastigmine. Several triterpenes have also been shown to act as inhibitors of AChE; this includes several hopanes [22], lanostanes [23] and lupanes [24]. Pentacyclic triterpenoic acids and their derivatives have been shown to be potent cholinesterase inhibitors in micro-molar range [25] with compounds of the α- or β-amyrin type being most active. Ursolic acid, for instance, acts as mixed-type inhibitor on AChE in the same magnitude as tacrine, a well-established drug [26]. Oleanolic acid [27,28] and structurally related compounds, *e.g.* taraxerol [29], echinocystic acid [30] or glycyrrhetinic acid [31], possessed IC_{50} values and inhibition constants K_i comparable to those of standard remedies like galantamine or donepezil.

Several studies an anti-tumor [32,33], anti-inflammatory [34] or an anti-viral [35] activity of maslinic acid and derivatives have been performed but the ability of these compounds to act as inhibitors of cholinesterases has not been investigated so far. Thus we prepared a series of maslinic acid derivatives differing in the substitution pattern at positions C-2, C-3 and C-28. All of these derivatives were screened for their ability to inhibit AChE and BChE; they were tested employing Ellman's assay, and their inhibitory constants (K_i and K_i') as well as the type of inhibition was determined. Furthermore, seven representative compounds were selected and investigated for a selectivity towards others enzymes [lipase from *Candida antarctica* (a serine hydrolase), papain (a sulfhydryl enzyme) and carbonic anhydrase II (a metalloenzyme)]. Additionally, some preliminary toxicity studies for selected derivatives were performed employing murine embryonic fibroblasts (NiH 3T3) in a photometric sulforhodamine B (SRB) assay.

2. Results and discussion

2.1. Chemistry

The first group of compounds (**1**–**27**, Scheme 1), representing several esters of maslinic acid, could be obtained from maslinic acid (**MA**) by reaction of **MA** with alkyl bromides in the presence of powdered K_2CO_3 in dry DMF [36,37].

The second group of compounds (Scheme 2) consists of matching pairs of maslinic acid derivatives (**28**, **29**), its methyl ester (**30**, **31**) and amides (**32**–**43**) possessing either free or acetylated hydroxyl groups at positions C-2 and C-3. Acetylations were carried out with acetic anhydride in pyridine [37] while the sulfamates **30** and **31** were obtained from the methyl ester **1** and sodium hydride in THF, followed by the addition of sulfamoyl chloride [39]. The synthesis of amides started from 2,3-diacetyl maslinic acid that was allowed to react with thionyl chloride followed by the addition of an amine [37].

A third group of compounds included augustic acid (**44**) and derivatives thereof. Augustic acid (**44**, Scheme 3) was synthesized *via* a four step chromatography-free synthesis starting from oleanolic acid as previously reported [39]. Its derivatizations were performed using well-established reactions as described above for the synthesis of derivatives of **MA**.

2.2. Biology

All compounds **1**–**48**, including maslinic acid (**MA**) and augustic acid (**44**), were subjected to Ellman's assays to determine their inhibitory activity (expressed as inhibition constants K_i and K_i') for the enzymes AChE and BChE. The results of these measurements are compiled in Table 1.

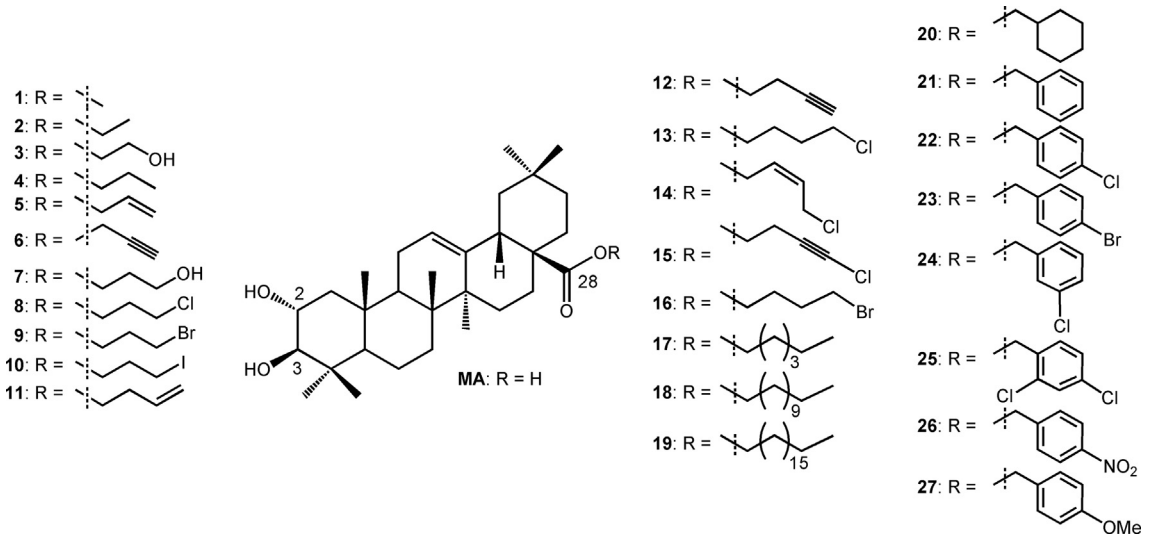
In summary, inhibitions constants for 24 compounds towards AChE and for 3 compounds towards BChE were determined. Two compounds, **18** and **19**, however, were not soluble under the conditions of the assay. For parent compound maslinic acid no inhibition constants below 100 μM could be obtained. Hence, maslinic acid does not significantly inhibit the cholinesterases; higher concentration could not be applied due to solubility reasons. Also, augustic acid (**44**) is no inhibitor of AChE, but – in contrast to maslinic acid – for BChE a mixed-type inhibition was observed (inhibition constants: K_i = 35.64 ± 5.73 μM and K_i' = 10.58 ± 1.95 μM). The uncompetitive part of the mixed-type inhibition (as expressed by K_i') is predominant. This indicates that augustic acid deploys its inhibitory action predominantly by binding to the enzyme–substrate complex rather than by binding to the free enzyme.

The first group of compounds (the esters **1**–**27**) were inhibitors of AChE, with the 1-chloro-butylester **13** as the most active compound of this series. This compound is a competitive inhibitor of AChE (K_i = 1.68 ± 0.30 μM). In comparison, for the esters **1**–**12** and **14**–**27** inhibition constants between 2.03 and 34.85 μM were determined. Especially, those esters having alkyl groups with more than three carbons showed K_i < 10 μM. While compounds **13** and **27** were competitive inhibitors, all other compounds of this series gave a mixed-type inhibition. Thus, the propyl ester **4** and the heptyl ester **17** gave an almost non-competitive inhibition (similar K_i and K_i') while a mixed-type inhibition with a dominating competitive part (K_i < K_i') could be determined for the ethyl ester **2** or the 1′-butinyl ester **12**. The 1-chloro-propyl ester **8** and the cyclohexyl ester **20** represent examples for mixed-type inhibitors with a dominating uncompetitive part (K_i > K_i').

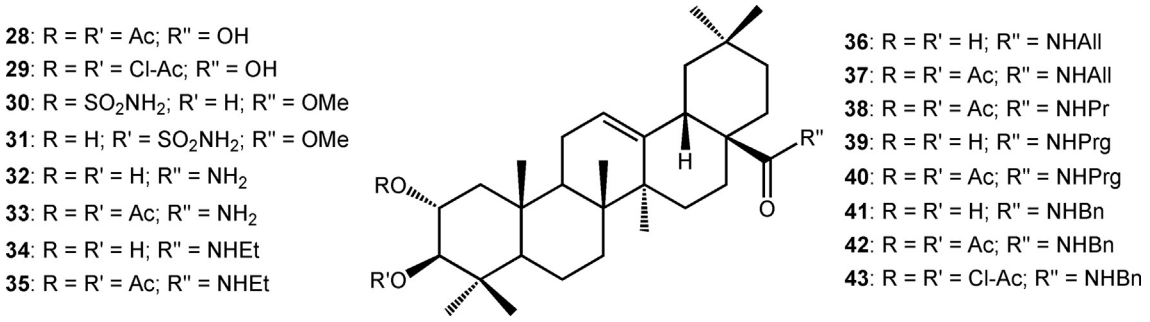
From the second group [representing 2,3-substituted maslinic acid esters (**28**–**31**) and amides (**32**–**43**)] four compounds (**29**, **31**, **40** and **43**) exhibited an activity towards AChE, only – albeit in moderate micro-molar magnitude. Thus, **29** and **31** showed a moderate mixed-type inhibition (K_i and K_i' between 10 and 40 μM) while the 2,3-diacetylated propargyl amide **40** was determined to act as a competitive inhibitior of AChE, and the amide **32** is a competitive inhibitor of BChE with an inhibition constant of K_i = 18.11 ± 3.43 μM. Out of this series, two compounds (**32** and **42**) were identified as inhibitors of BChE. Their inhibitory activity is slightly lower than that of standard galantamine hydrobromide (K_i = 9.37 ± 0.67 μM).

As far as the last group of compounds [consisting of augustic acid (**44**) and related derivatives **44**–**48**] is concerned, parent augustic acid turned out to be a mixed-type inhibitor of BChE with a dominating uncompetitive part (K_i = 35.64 ± 5.73 μM, K_i' = 10.58 ± 1.95 μM); this compound is no inhibitor of AChE. Compounds **45** and **46** did not show any activity for AChE or BChE, while 2,3-dichloroacetyl-substituted **47** and **48** act as AChE inhibitors. Both compounds are mixed-type inhibitors in the single-digit micro-molar range.

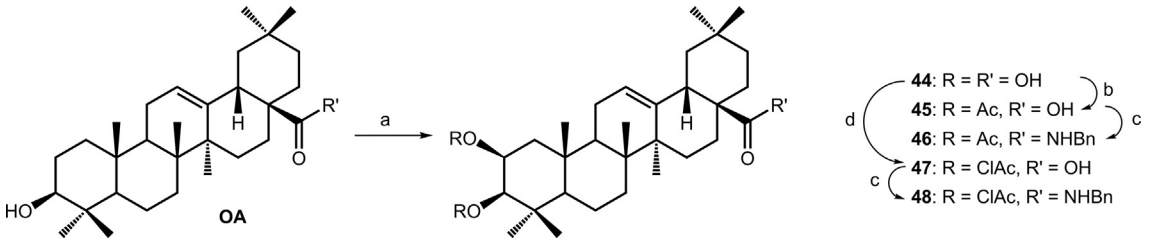
Another important criterion is the selectivity of the compounds concerning one of the cholinesterases on one hand and concerning



Scheme 1. Structures of maslinic acid (**MA**) and esters **1**–**27**.



Scheme 2. Structures of 2,3-substituted maslinic acid esters **28**–**31** and amides **32**–**43** (All = allyl, Prg = propargyl).



Scheme 3. Synthesis of augustic acid (**44**) and related derivatives (**45**–**48**); a) ref 38; b) acetanhydride, triethylamine, DMAP, DCM, r.t., 1 day; c) I. $(COCl)_2$, DCM, triethylamine, DMF, r.t., 2 h – benzylamine, triethylamine, DMAP, DCM, 0 °C, 5 min; d) chloroacetic anhydride, triethylamine, DMAP, DCM, r.t., 1 day.

other enzymes on the other. Compounds showing the highest selectivity (expressed by F = K_i of AChE divided by K_i of BChE) towards AChE are compounds **10** (F < 0.3), **13** (F < 0.17) and **14** (F < 0.28). Both most active BChE inhibitors **32** (F > 1.10) and **42** (F = 1.30) showed a slight selectivity towards BChE.

In addition, seven representative compounds were selected and subjected to assays (Table 2) employing bovine carbonic anhydrase, the lipase from *C. antarctica* and papain from *Carica papaya*. Each of these enzymes represents a different mechanism in the active site of the enzyme. All derivatives showed only a low or no inhibition at all - even at the maximum concentration used in the assays. Only compounds **9**, **10** and **14** showed a marginal impact on these enzymes.

A low cytotoxicity is mandatory for compounds intended for the treatment of AD. Thus, IC_{50} were measured utilizing a photometric sulforhodamine B assay employing murine embryonic fibroblasts (NiH 3T3) [36,37]; the results of these assays are compiled in Table 3. While parent maslinic acid showed an IC_{50} value of 16.6 μM, cytotoxicity decreased for some of the compounds: *e.g.* for **9** (IC_{50} = 21.6 μM) or **14** (IC_{50} = 33.1 μM). Moreover, the cytotoxicity of all derivatives was significantly lower than their inhibition constants, except for the propyl ester **4** and the 1-bromopropyl ester **9**.

2.3. Docking studies

Molecular modeling studies were performed to evaluate the molecular features being important for the inhibitory activity against AChE of derivatives of **MA**, to gain some insights on their mode of action and to explain their different kinetic behavior. For

Table 1
Inhibitory constants for galantamine hydrobromide (**GH**), **MA** and compounds **1**–**54** (K_i and K_i' in μM), determined using Ellman's assay employing acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) with galantamine hydrobromide as standard. Four different substrate concentrations and four different inhibitor concentrations were used; each experiment was performed at least in triplicate; n. sol – not soluble.

	AChE	AChE	BChE		AChE	AChE	BChE	BChE
	K_i	K_i'	K_i		K_i	K_i'	K_i	K_i'
GH	0.54 ± 0.01		9.37 ± 0.67	**24**	>10		>10	
MA	>100		>100	**25**	2.03 ± 0.01	9.24 ± 0.93	>6	
1	>50		>50	**26**	6.14 ± 0.28	7.22 ± 0.69	>4	
2	3.49 ± 0.71	4.67 ± 0.25	>14	**27**	6.49 ± 1.68	69.56 ± 0.14	>100	
3	>100		>100	**28**	>10		>10	
4	16.86 ± 3.31	15.40 ± 1.14	>6	**29**	13.12 ± 1.02	19.77 ± 0.44	>20	
5	>30		>30	**30**	>40		>40	
6	>10		>10	**31**	20.53 ± 7.47	38.58 ± 3.11	>20	
7	20.12 ± 0.96	13.73 ± 0.12	>10	**32**	>20		18.11 ± 3.43	
8	8.76 ± 2.02	3.45 ± 0.24	>6	**33**	>20		>20	
9	34.85 ± 2.06	24.43 ± 0.23	>80	**34**	>20		>20	
10	6.01 ± 0.55	13.44 ± 3.19	>20	**35**	>14		>14	
11	3.31 ± 0.81	11.51 ± 0.31	>10	**36**	>20		>20	
12	6.76 ± 0.95	32.51 ± 2.41	>20	**37**	>20		>20	
13	1.68 ± 0.30		>10	**38**	>6		>6	
14	22.48 ± 1.70	9.38 ± 0.80	>80	**39**	>20		>20	
15	5.71 ± 0.71	14.83 ± 1.66	>6	**40**	8.14 ± 1.85		>14	
16	>10		>10	**41**	>10		>10	
17	4.78 ± 1.63	5.11 ± 0.75	>6	**42**	16.19 ± 1.09	39.44 ± 2.71	12.43 ± 1.58	45.44 ± 3.43
18	n. sol.			**43**	25.14 ± 4.39	31.50 ± 2.53	>30	
19	n. sol.			**44**	>30		35.64 ± 5.73	10.58 ± 1.95
20	10.02 ± 0.47	2.67 ± 0.20	>14	**45**	>20		>20	
21	>14		>14	**46**	>10		>10	
22	>30		>30	**47**	6.85 ± 0.47	9.36 ± 0.18	>6	
23	>20		>20	**48**	6.25 ± 0.77	16.26 ± 0.40	>6	

Table 2
Relative inhibition (in %) of the enzymes AChE, BChE, carbonic anhydrase II (from bovine erythrocytes), lipase (from *Candida antarctica*) as well as papain (from *Carica papaya*) by compounds **4**, **9**, **10**, **13**, **14**, **17** and **25**. Concentration (in μM) represents the maximum concentration for each compound in the assay. Experiments were performed at least in triplicate.

Compound	**4**	**9**	**10**	**13**	**14**	**17**	**25**
conc. [μM]	3	40	10	5	40	3	3
AChE	56	39	51	79	79	62	43
BChE	0	16	0	0	7	0	0
bCA II	0	1	6	0	1	0	0
Lipase	0	18	0	0	0	0	0
Papain	0	0	0	0	0	0	0

Table 3
Cytoxicity [IC_{50} values in μM [36,37] from photometric SRB assays, using non-malignant murine embryonic fibroblasts (NiH 3T3)] and inhibition constants K_i. (in μM for AChE) for selected compounds. Each experiment was performed at least in triplicate.

Compound	MA	**4**	**9**	**10**	**13**	**14**	**17**	**25**
IC_{50}	16.6	13.4	21.6	12.3	12.9	33.1	14.1	17.3
K_i (AChE)	>100	16.86	34.85	6.01	1.68	22.48	4.78	2.03

example, closely related compounds exhibited different kinetic behavior and were shown to be inhibitors in a single digit micromolar concentration: Compound **27** was shown to be a competitive inhibitor, while **12** inhibited AChE in a mixed-type manner with a dominating competitive part ($K_i < K_i'$), and **20** was a mixed-type inhibitor with a dominating uncompetitive part ($K_i > K_i'$**0**). The coordinates of the enzyme structure were obtained from the Protein Data Bank (accession code 4BDT), and the docking experiments of the maslinic acid derivatives **12**, **20** and **27** were performed using GOLD 5.2 software [40,41]. We decided to use the final target hAChE for the docking studies as no differences are observed in the active site gorge for the different organisms. In addition, the homology between EeAChE and hAChE is very high (88%) and no significant differences are observed in the active site. Comparison of the docked pose for the competitive inhibitor **27** (green, Fig. 1) with the poses for the mixed-type inhibitors **12** and **20** (cyano and pink, respectively, Fig. 1) showed inhibitor **27** entering deep into the active-site gorge and performing H-π and H–C=O weak interactions with Tyr449 and the catalytic His447. This might explain its competitive character. In addition, a 2-OH-π interaction is observed with the Trp286 in the entrance region of the gorge. On the other hand, the most favorable poses for the mixed-type inhibitors **12** and **20** suggest that those do not enter the active-site gorge. However, for each compound two CH-π interactions were observed with the Trp286 at the entrance of the gorge, thus indicating that the complex with these inhibitors forms an active site gorge lid leading to mixed-type inhibitors. Nevertheless the docking results do not give a molecular insight to explain the different competitive/uncompetitive character for mixed-type inhibitors **12** and **20**, respectively.

3. Conclusion

In this study 43 derivatives of maslinic acid as well as five compounds derived from augustic acid were synthesized and subjected to Ellman's assay to determine their potential as inhibitors of cholinesterases. While no inhibition could be detected for parent maslinic acid, 22 of its derivatives inhibited AChE, and two compounds were inhibitors for BChE in a micromolar range. Augustic acid was an inhibitor of BChE inhibitor, whereas some of its derivatives inhibited AChE. A chloro-butylester of maslinic acid **13** was the most active species of this study possessing an inhibition constant of $K_i = 1.68$ μM; it is a competitive inhibitor for AChE. However, almost all of the active compounds were mixed-type inhibitors, and small differences in their mode of inhibitory action were detected, since the ratios of the inhibition constants K_i

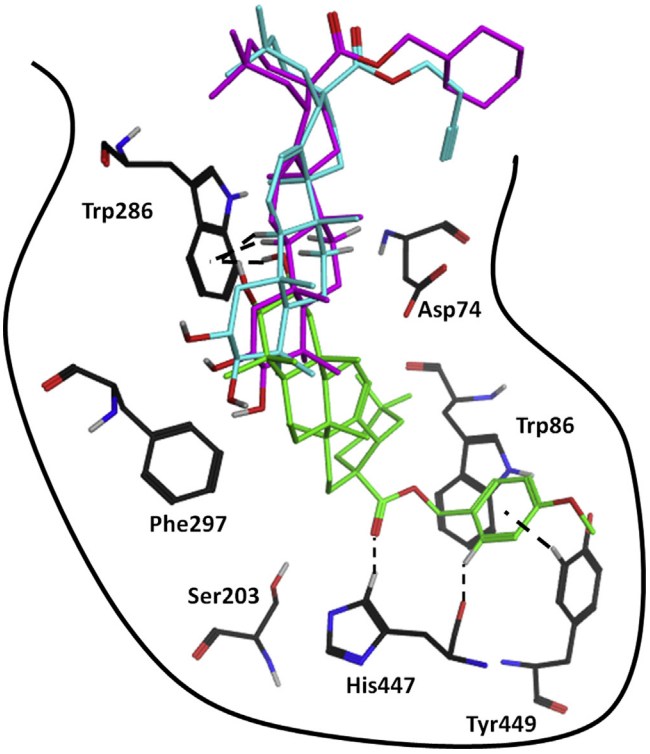


Fig. 1. Docked poses for compounds **12** (cyano), **20** (pink) and **27** (green) in the AChE active site. AChE PDB code 4BDT was used for docking with GOLD 5.2 software [40]. Image prepared using MOE2013 [41]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and K_i' varied. Some of the compounds were mixed-type inhibitors with a dominating competitive part ($K_i < K_i'$, *e.g.* for **2** and **12**), and a dominating uncompetitive part ($K_i > K_i'$) was determined for four derivatives and, finally, a nearly non-competitive inhibition ($K_i \approx K_i'$, *e.g.* for **4** and **17**) was found, as well.

Docking studies revealed that the different kinetic behavior for very similar compounds can be explained by the ability of the compounds to enter the active site gorge of AChE. The competitive inhibitor **27** was able to enter deep into the gorge and to interact with the catalytic machinery of AChE while the mixed-type inhibitors **12** and **20** interacted with the enzyme's Trp286 at the gorge entrance indicating that these inhibitors may form a active-site lid that governs the inhibiton of AChE.

Selectivity and cytotoxicity studies for selected compounds showed the potential of this compounds as to act as selective inhibitors for cholinesterases. Consequently, we were able to convert maslinic acid into potent inhibitors of cholinesterases thus disclosing a new field of application for this triterpenoic acid, and qualifying derivatives thereof for further biological studies in the field of dementia and AD research.

4. Experimental part

4.1. Chemistry

4.1.1. General

The reagents were bought from commercial suppliers without any further purification. NMR spectra were measured on VARIAN Gemini 2000 or Unity 500 spectrometers at 27 °C with trimethylsilane as an internal standard, δ are given in ppm and J in Hertz. Mass spectra were taken on a FINNIGAN LCQ instrument. IR spectra were recorded on a Perkin–Elmer FT-IR spectrometer Spectrum 1000, optical rotations on a Perkin–Elmer 341 polarimeter (1 cm micro cell) and UV–vis spectra on a Perkin–Elmer unit, Lambda 14. Melting points were measured with a LEICA hot stage microscope and are uncorrected. Elemental analysis was done on a Foss-Heraeus Vario EL unit. TLC was performed on silica gel (Merck 5554, detection by UV absorption). Solvents were dried before use according to usual procedures. The purity of the compounds was shown to be >98% (by HPLC). Maslinic and augustic acid were prepared from oleanolic acid as previously described [38]. The following compounds were synthesized as previously published: **1**–**19**, **28** and **32**–**40** [37], **20**–**27**, **41** and **42** [36], and **30** and **31** [39].

4.1.2. (2α,3β) 2,3-Bis(chloroacetyloxy)-olean-12-en-28-oic acid (*29*)

Maslinic acid (200 mg, 0.42 mmol), triethylamine (0.16 mL, 1.15 mmol) and catalytic amounts of DMAP were dissolved in dry DCM (50 mL). A solution of chloroacetic anhydride (0.26 mg, 1.53 mmol) in dry DCM (10 mL) was slowly added, and stirring at 25 °C was continued for another 30 min. The reaction was quenched with by adding 2 N HCl and extracted with Et_2O (3 × 25 mL). The combined organic layers were washed with brine (20 mL), filtered, dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was subjected to column chromatography (silica gel, hexane/ethyl acetate, 7:3) to afford **29** (198 mg, 75%) as a colorless solid; m.p. 239–243 °C; $R_F = 0.38$ (silica gel, hexane/ethyl acetate, 7:3); $[\alpha]_D = 22.52°$ ($c = 0.29$, $CHCl_3$); IR (KBr): ν = 3420*s*, 2949*s*, 1743*s*, 1695*s*, 1463*m*, 1410*m*, 1397*m*, 1309*s*, 1180*s*, 1025*s*, 994*s*, 970*m*, 924*m*, 823*s*, 791*s*, 718*m*, 651*s*, 572 *s* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.25$ (dd, $J = 3.4$, 3.4 Hz, 1H, C*H* (12)), 5.17 (ddd, $J = 11.1$, 10.8, 4.7 Hz, 1H, C*H* (2)), 4.85 (d, $J = 10.3$ Hz, 1H, C*H* (3)), 4.03 (s, 1H, CH_a (33)), 4.02 (s, 1H, CH_b (33)), 3.95 (s, 2H, CH_2 (32)), 2.81 (dd, $J = 13.7$, 4.1 Hz, 1H, C*H* (18)), 2.06 (dd, $J = 12.4$, 4.7 Hz, 1H, CH_a (1)), 2.01–1.92 (m, 1H, CH_a (16)), 1.92–1.87 (m, 1H, CH_a (11)), 1.87–1.79 (m, 1H, CH_b (11)), 1.79–1.65 (m, 2H, CH_a (22) + CH_a (15)), 1.65–1.51 (m, 5H, C*H* (9) + CH_a (19) + CH_b (22) + CH_b (16) + CH_a (6)), 1.51–1.42 (m, 1H, CH_a (7)), 1.39–1.28 (m, 3H, CH_b (6) + CH_a (21) + CH_b (7)), 1.28–1.16 (m, 1H, CH_b (21)), 1.15–1.09 (m, 5H, CH_b (19) + CH_b (1) + CH_3 (27)), 1.09–1.02 (m, 4H, CH_b (15) + CH_3 (25)), 1.01–0.94 (m, 1H, C*H* (5)), 0.92 (s, 3H, CH_3 (29)), 0.91 (s, 3H, CH_3 (23)), 0.91 (s, 3H, CH_3 (24)), 0.89 (s, 3H, CH_3 (30)), 0.72 (s, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 184.6 (*C*=O, C28), 167.3 (*C*=O, C31), 167.0 (*C*=O, C34), 143.8 ($C_{quart.}$, C13), 122.1 (H*C*=C, C12), 82.3 (*C*H, C3), 72.21 (*C*H, C2), 54.9 (*C*H, C5), 47.6 (*C*H, C9), 46.6 ($C_{quart.}$, C17), 45.9 (CH_2, C19), 43.6 (CH_2, C1), 41.7 ($C_{quart.}$, C14), 41.0 (CH_2, C32), 41.0 (*C*H, C18), 40.9 (CH_2, C33), 39.7 ($C_{quart.}$, C4), 39.4 ($C_{quart.}$, C8), 38.4 ($C_{quart.}$, C10), 33.9 (CH_2, C21), 33,2 (CH_3, C30), 32.5 (CH_2, C22), 32.4 (CH_2, C7), 30.8 ($C_{quart.}$, C20), 28.4 (CH_3, C23), 27.7 (CH_2, C15), 26.0 (CH_3, C27), 23.7 (CH_3, C29), 23.5 (CH_2, C11), 22.9 (CH_2, C16), 18.2 (CH_2, C6), 17.6 (CH_3, C24), 17.2 (CH_3, C26), 16.5 (CH_3, C25) ppm; MS (ESI): *m/z* (%) = 623.4 ($[M-H]^-$, 100), 1249.1 ($[2M-H]^-$, 94); anal. calcd for $C_{34}H_{50}Cl_2O_6$ (625.66): C 65.27, H 8.05; found: C 65.01, H 8.14.

4.1.3. Benzyl (2α,3β) 2,3-bis(chloroacetyloxy)-olean-12-en-28-amide (*43*)

To a solution of **29** (100 mg, 0.16 mmol) in dry dichloromethane (20 mL), containing 2 drops of dry DMF and trimethylamine, at 0 °C oxalyl chloride (2 mL, 0.023 mol) was added, and stirring at room temperature was continued for 1 h. The solvents were removed under reduced pressure, dry THF (20 mL) added, and the mixture was concentrated again. The residue was dissolved in dry DCM (15 mL), and benzylamine (1.3 mL, 0.012 mol), 2 drops of triethylamine and catalytic amounts of DMAP were added. After stirring at room temperature for 15 min, the reaction was quenched by adding 2 N hydrochloric acid (10 mL) and extracted with Et_2O (3 × 15 mL).

The combined organic layers were washed with brine (10 mL), filtered, dried ($MgSO_4$) and evaporated to dryness. Purification by column chromatography (silica gel, hexane/ethyl acetate, 8:2) gave **43** (62 mg, 54%) as a colorless solid; m.p. 117–119 °C; R_F = 0.38 (silica gel, hexane/ethyl acetate, 8:2); $[\alpha]_D$ = −5.04° (*c* = 0.28, $CHCl_3$); IR (KBr): ν = 3432*m*, 2948*s*, 1744*s*, 1650*m*, 1517*m*, 1454*s*, 1396*w*, 1309*s*, 1264*s*, 1185*s*, 1025*m*, 1001*s*, 751*s*, 699 *s* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.40–7.20 (*m*, 5H, CH_a (37) + CH_b (37) + CH_a (38) + CH_b (38) + CH (39)), 6.16 (*dd*, *J* = 5.2, 5.2 Hz, 1H, N*H*), 5.31 (*dd*, *J* = 3.2, 3.2 Hz, 1H, C*H* (12)), 5.18 (*ddd*, *J* = 11.2, 11.0, 4.5 Hz, 1H, C*H* (2)), 4.86 (*d*, *J* = 10.3 Hz, 1H, C*H* (3)), 4.61 (*dd*, *J* = 14.6, 6.1 Hz, 1H, CH_a (35), 4.17 (*dd*, *J* = 14.6, 4.4 Hz, 1H, CH_b (35)), 4.05 (*s*, 1H, CH_a (32)), 4.05 (*s*, 1H, CH_b (32)), 3.96 (*s*, 2H, CH_2 (33)), 2.57 (dd, *J* = 12.7, 3.3 Hz, 1H, C*H* (18)), 2.12–2.03 (m, 1H, CH_a (1)), 2.03–1.93 (*m*, 1H, CH_a (16)), 1.84 (*m*, *J* = 8.7, 3.1 Hz, 2H, CH_a (11) + CH_b (11)), 1.81–1.53 (*m*, 7H, CH_a (19) + CH_a (22) + CH_b (16) + CH_b (22) + C*H* (9) + CH_a (15) + CH_a (6)), 1.53–1.18 (*m*, 5H, CH_a (7) + CH_b (6) + CH_a (21) + CH_b (7) + CH_b (21)), 1.17–1.12 (*m*, 4H, CH_b (19) + CH_3 (27)), 1.12–1.05 (*m*, 2H, CH_b (1) + CH_b (15)), 1.03 (s, 3H, CH_3 (25)), 1.01–0.96 (*m*, 1H, C*H* (5)), 0.94 (*s*, 3H, CH_3 (24)), 0.94 (*s*, 3H, CH_3 (23)), 0.92 (*s*, 3H, CH_3 (30)), 0.92 (*s*, 3H, CH_3 (29)), 0.66 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.9 (*C*=O, C28), 167.3 (*C*=O, C31), 167.0 (*C*=O, C34), 145.1 (HC=*C*, C13), 138.5 (*C*H_{Ar}, C36), 128.8 (*C*H_{Ar}, C38), 127.9 (*C*H_{Ar}, C37), 127.5 (*C*H_{Ar}, C39), 122.1 (H*C*=C, C12), 82.3 (*C*H, C3), 72.2 (*C*H, C2), 54.9 (*C*H, C5), 47.5 (*C*H, C9), 46.7 (*C*H_2, C19), 46.4 ($C_{quart.}$, C17), 43.7 (*C*H_2, C1), 43.7 (*C*H_2, C35), 42.4 (*C*H, C18), 42.2 ($C_{quart.}$, C14), 41.0 (*C*H_2, C32), 40.9 (*C*H_2, C33), 39.7 ($C_{quart.}$, C4), 39.5 ($C_{quart.}$, C8), 38.3 ($C_{quart.}$, C10), 34.2 (*C*H_2, C21), 33.1 (*C*H_3, C30), 32.8 (*C*H_2, C22), 32.2 (*C*H_2, C7), 30.9 ($C_{quart.}$, C20), 28.4 (*C*H_3, C23), 27.4 (*C*H_2, C15), 25.8 (*C*H_3, C27), 23.8 (*C*H_2, C16), 23.7 (*C*H_3, C29), 23.6 (*C*H_2, C11), 18.2 (*C*H_2, C6), 17.6 (*C*H_3, C24), 17.0 (*C*H_3, C26), 16.5 (*C*H_3, C25) ppm; MS (ESI): *m/z* (%) = 714.3 ($[M+H]^+$, 100), 736.1 ($[M+Na]^+$, 26), 1429.9 ($[2M+H]^+$, 32), 1451.3 ($[2M+Na]^+$, 52); anal. calcd for $C_{41}H_{57}Cl_2NO_5$ (714.80): C 68.89, H 8.04, N 1.96; found: C 68.69, H 8.15, N 1.77.

4.1.4. (2β,3β) 2,3-Bis(2-chloroacetoxy)-olean-12-en-28-oic acid (**47**)

To a solution of compound **44** (200 mg, 0.42 mmol) in dry dichloromethane (20 mL), chloroacetic acid (0.20 mL, 1.69 mmol), triethylamine (0.25 mL, 1.80 mmol) and DMAP (5 mg, 0.04 mmol) were added, and stirring was continued for one day. Usual aqueous work-up followed by chromatography (silica gel, hexane/ethyl acetate, 8:2) yielded **47** (170 mg, 64%) as a colorless solid; m.p. 272–274 °C; R_F = 0.27 (silica gel, hexane/ethyl acetate, 8:2); $[\alpha]_D$ = 80.95° (*c* = 0.32, $CHCl_3$); IR (KBr): ν = 2950*vs*, 1764*s*, 1740*vs*, 1698*s*, 1462*m*, 1306*s*, 1288*s*, 1262*m*, 1180*s*, 1154*s*, 996 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.46–5.43 (*m*, 1H, C*H* (2)), 5.28 (*dd*, *J* = 3.6, 3.6 Hz, 1H, C*H* (12)), 4.73 (*d*, *J* = 3.9 Hz, 1H, C*H* (3)), 4.04–4.03 (*m*, 4H, CH_2 (32) + CH_2 (34)), 2.83 (*dd*, *J* = 13.7, 4.3 Hz, 1H, C*H* (18)), 2.04 (*dd*, *J* = 15.2, 3.0 Hz, 1H, CH_a (1)), 2.01–1.94 (*m*, 2H, CH_a (11) + CH_a (16)), 1.89–1.68 (*m*, 3H, CH_b (11) + CH_a (22) + CH_a (15)), 1.65–1.44 (*m*, 7H, CH_2 (6) + CH_b (16) + CH_a (19) + CH_b (22) + C*H* (9) + CH_a (7)), 1.43–1.29 (*m*, 3H, CH_b (7) + CH_a (21) + CH_b (1)), 1.25–1.12 (*m*, 2H, CH_b (21) + CH_b (19)), 1.22 (*s*, 3H, CH_3 (25)), 1.13 (*s*, 3H, CH_3 (27)), 1.10–1.04 (*m*, 1H, CH_b (15)), 1.08 (*s*, 3H, CH_3 (23)), 1.03–0.98 (*m*, 1H, C*H* (5)), 0.94 (*s*, 3H, CH_3 (24)), 0.93 (*s*, 3H, CH_3 (29)), 0.90 (*s*, 3H, CH_3 (30)), 0.77 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 184.3 (*C*=O, C28), 167.1 (*C*=O, C33), 167.0 (*C*=O, C31), 144.0 (*C*=CH, C13), 122.4 (H*C*=C, C12), 80.0 (*C*H, C2), 71.5 (*C*H, C3), 55.3 (*C*H, C5), 48.2 (*C*H, C9), 46.7 ($C_{quart.}$, C17), 45.9 (*C*H_2, C19), 41.9 (*C*H_2, C1), 41.9 ($C_{quart.}$, C14), 41.1 (*C*H, C18), 41.0 (*C*H_2, C32), 41.0 (*C*H_2, C34), 39.5 ($C_{quart.}$, C8), 37.6 ($C_{quart.}$, C4), 36.8 ($C_{quart.}$, C10), 33.9 (*C*H_2, C21), 33.2 (*C*H_3, C30), 32.6 (*C*H_2, C22), 32.6 (*C*H_2, C7), 30.8 ($C_{quart.}$, C20), 29.2 (*C*H_3, C24), 27.7 (*C*H_2, C15), 26.2 (*C*H_3, C27), 23.7 (*C*H_3, C29), 23.6 (*C*H_2, C11), 22.9 (*C*H_2, C16), 18.0 (*C*H_2, C6), 17.7 (*C*H_3, C23), 17.4 (*C*H_3, C26), 16.0 (*C*H_3, C25) ppm; MS (ESI): *m/z* (%) = 623.4 ($[M-H]^-$, 74), 1249.3 ($[2M-H]^-$, 100); anal. calcd for $C_{34}H_{50}Cl_2O_6$ (625.66): C 65.27, H 8.05; found: C 65.05, H 7.87.

4.1.5. Benzyl (2β,3β) 2,3-bis(2-chloroacetyloxy)-olean-12-en-28-amide (**48**)

Following the procedure given for the synthesis of **46**, from **47** (110 mg, 0.18 mmol) **48** (100 mg, 80%) was obtained as a colorless solid; m.p. 115–119 °C; R_F = 0.30 (silica gel, hexane/ethyl acetate, 8:2); $[\alpha]_D$ = 41.28° (*c* = 0.33, $CHCl_3$); IR (KBr): ν = 2948*vs*, 2868*m*, 1762*s*, 1740*vs*, 1654*s*, 1516*s*, 1454*m*, 1290*s*, 1262*s*, 1184*s*, 1154*s*, 994*m*, 698 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.34–7.30 (*m*, 2H, C*H* (38)), 7.28–7.23 (*m*, 3H, C*H* (37) + C*H* (39)), 6.15 (*dd*, *J* = 6.1, 4.7 Hz, 1H, N*H*), 5.44–5.41 (*m*, 1H, C*H* (2)), 5.30 (*dd*, *J* = 3.6, 3.6 Hz, 1H, C*H* (12)), 4.71 (*d*, *J* = 3.9 Hz, 1H, C*H* (3)), 4.60 (*dd*, *J* = 14.7, 6.3 Hz, 1H, CH_a (35)), 4.16 (*dd*, *J* = 14.7, 4.5 Hz, 1H, CH_b (35)), 4.05–4.03 (*m*, 4H, CH_2 (32) + CH_2 (34)), 2.55 (*dd*, *J* = 13.1, 4.0 Hz, 1H, C*H* (18)), 2.04–1.95 (*m*, 2H, CH_a (1) + CH_a (16)), 1.92–1.78 (*m*, 2H, CH_2 (11)), 1.79–1.70 (*m*, 2H, CH_a (22) + CH_a (19)), 1.70–1.43 (*m*, 7H, CH_2 (6) + CH_b (16) + CH_a (15) + CH_b (22) + C*H* (9) + CH_a (7)), 1.40–1.32 (*m*, 2H, CH_b (1) + CH_a (21)), 1.32–1.13 (*m*, 3H, CH_b (21) + CH_b (19) + CH_b (7)), 1.16 (*s*, 3H, CH_3 (25)), 1.15 (*s*, 3H, CH_3 (27)), 1.08 (*s*, 3H, CH_3 (23)), 1.06–1.00 (*m*, 1H, CH_b (15)), 0.99–0.95 (*m*, 1H, C*H* (5)), 0.93 (*s*, 3H, CH_3 (24)), 0.90 (*s*, 3H, CH_3 (30)), 0.90 (*s*, 3H, CH_3 (29)), 0.69 (*s*, 3H, CH_3 (26)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.0 (*C*=O, C28), 167.0 (*C*=O, C33), 167.0 (*C*=O, C31), 145.2 (*C*=CH, C13), 138.5 (*C*$_{Ar}$, C36), 128.8 (*C*H_{Ar}, C38), 127.9 (*C*H_{Ar}, C37), 127.5 (*C*H_{Ar}, C39), 122.5 (H*C*=C, C12), 79.9 (*C*H, C2), 71.5 (*C*H, C3), 55.2 (*C*H, C5), 48.1 (*C*H, C9), 46.7 (*C*H_2, C19), 46.5 ($C_{quart.}$, C17), 43.7 (*C*H_2, C35), 42.4 (*C*H, C18), 42.3 ($C_{quart.}$, C14), 41.9 (*C*H_2, C1), 41.0 (*C*H_2, C32), 41.0 (*C*H_2, C34), 39.6 ($C_{quart.}$, C8), 37.6 ($C_{quart.}$, C4), 36.6 ($C_{quart.}$, C10), 34.2 (*C*H_2, C21), 33.1 (*C*H_3, C30), 32.8 (*C*H_2, C22), 32.4 (*C*H_2, C7), 30.8 ($C_{quart.}$, C20), 29.1 (*C*H_3, C24), 27.3 (*C*H_2, C15), 25.9 (*C*H_3, C27), 23.9 (*C*H_2, C16), 23.7 (*C*H_3, C29), 23.6 (*C*H_2, C11), 18.0 (*C*H_2, C6), 17.7 (*C*H_3, C23), 17.1 (*C*H_3, C26), 16.1 (*C*H_3, C25) ppm; MS (ESI): *m/z* (%) = 714.3 ($[M+H]^+$, 100), 736.3 ($[M+Na]^+$, 16), 1429.1 ($[2M+H]^+$, 52), 1451.2 ($[2M+Na]^+$, 22); anal. calcd for $C_{41}H_{57}Cl_2NO_5$ (714.80): C 68.89, H 8.04, N 1.96; found: C 68.71, H 8.21, N 1.72.

4.2. Biology

4.2.1. Cell lines and culture conditions

The NiH 3T3 cells were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

4.2.2. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (SRB) (Sigma Aldrich) microculture colorimetric assay as previously reported [36–38,42].

4.2.3. Enzymatic studies

4.2.3.1. Spectrophotometer and chemicals. A TECAN Spectra-FluorPlus instrument working on the kinetic mode and measuring the absorbance at λ = 415 nm was used for the enzymatic studies. Acetylcholinesterase (from *Electrophorus electricus*), Papain (from *Carica papaya*), Lipase (from *C. antarctica*), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide were purchased from Fluka. Butyrylcholinesterase (from equine serum), carbonic anhydrase II (from bovine erythrocytes) as well as 4-nitrophenyl acetate (4-NA) were purchased from Sigma, and

butyrylthiocholine idioide was bought from Aldrich.

4.2.3.2. Solutions preparation. Preparation of 50 mM Tris–HCl buffer solutions: Tris(hydroxymethyl)-aminomethane (606 mg) was dissolved in bidestilled water (100 mL) and the pH was adjusted with HCl to 8.0 ± 0.1 (for AChE, BChE and the lipase) and 6.2 ± 0.1 (for papain), respectively. Buffers were freshly prepared and stored in the refrigerator. AChE solution 2.005 U/ml: the enzyme (271 U/mg, 0.037 mg) was dissolved in freshly prepared buffer pH 8.0 (5 mL) containing NaN_3 (0.98 mg). BChE solution 2.040 U/ml: the enzyme (7.54 U/mg, 1.353 mg) was dissolved in freshly prepared buffer pH 8.0 (5 mL) containing NaN_3 (0.98 mg). Papain solution 3.889 U/ml: the enzyme (2.1 U/mg, 9.52 mg) was dissolved in freshly prepared buffer pH 6.2 (5 mL). Lipase solution 2.003 U/ml: the enzyme (3.1 U/mg, 3.23 mg) was dissolved in freshly prepared buffer pH 8.0 (5 mL). DTNB solution 3 mM: DTNB (23.8 mg) was dissolved in freshly prepared buffer pH 8.0 (20 mL) containing NaCl (116.8 mg) and $MgCl_2$ (38.0 mg). ATChI solution 15 mM: ATChI (43.4 mg) was dissolved in bidestilled water (10 mL). BTChI solution 15 mM: BTChI (47.6 mg) was dissolved in bidestilled water (10 mL). 4-NA solution 6 mM: 4-NA (21.6 mg) was dissolved in methanol (2.2 mg) and bidestilled water (17.8 mL). All solutions were stored in Eppendorf vials in the refrigerator or freezer, if necessary. The pure compounds were initially dissolved in DMSO, galantamine hydrobromide (as standard for AChE and BChE) was dissolved in bidistilled water. The final concentrations for the enzymatic assay were obtained by diluting the stock solution with bidistilled water. No inhibition was detected by residual DMSO (<0.5%).

4.2.3.3. Enzyme assay

4.2.3.3.1. Cholinesterase-assay. A mixture of the DTNB solution (125 μL), enzyme (25 μL) and compounds solutions (25 μL, 3 different concentrations and one blank with water) was prepared and incubated at 30 °C for 20 min. The substrate (25 μL, 4 different concentrations) was added to start the enzymatic reaction. The absorbance data (λ = 415 nm) was recorded at 30 °C for 30 min using 1 min intervals. All measurements were performed as triplicates. The substrate concentrations in the test were as follows: [ATChI] = [BTChI] = 0.9375 mM, 0.625 mM, 0.325 mM, 0.1875 mM. The mode of inhibition as well as K_i and K_i' were determined using Lineweaver-Burk plot, Dixon plot and Cornish-Bowden plot.

4.2.3.3.2. Lipase-assay. A mixture of buffer solution pH 8.0 (125 μL), enzyme (25 μL) and compounds solutions (25 μL, 50 μM) was prepared and incubated at 37 °C for 20 min. 4-NA (25 μL, 0.75 μM) was added to start the enzymatic reaction. The absorbance data (λ = 415 nm) was recorded at 37 °C for 10 min using 1 min intervals. All measurements were performed in duplicate. The relative inhibition was determined as the quotient of the slopes (compound divided by blank) of the linear ranges.

4.2.3.3.3. Papain-assay. The assay was performed as reported for the lipase-Assay but using a buffer solution of pH 6.2 instead.

4.2.4. Docking studies

The 3D structure coordinates of AChE were obtained from the Protein Data Bank, PDB code 4BDT (with 3.10 Å resolution). To prepare the enzyme for the docking studies, the co-crystallized inhibitor as well as crystallographic water molecules included in the PDB files were removed. Hydrogen atoms were added, and the protonation states were correctly assigned using the Protonate-3D tool within the Molecular Operating Environment (MOE) 2011.13 software package [40], and energy was minimized using a MMFF94x force field. Molecular docking studies were performed using the GoldScore scoring function from the GOLD 5.2 [39] software package, and each ligand was subjected to 500 docking runs, using Trp86 N atom as active site center coordinate. The docking radius was considered 15 Å from the active site center. The docking protocol was validated by the docking of the co-crystallized inhibitor in 4BDT; the RMSD value between docked and crystallographic poses was 1.27 Å.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.09.007.

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