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Probing the mid-gorge of cholinesterases with spacer-modified bivalent quinazolinimines leads to highly potent and selective butyrylcholinesterase inhibitors

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ABSTRACT

The spacer structure of homobivalent quinazolinimes acting as potent acetyl-(AChE)- and butyrylcholinesterase (BChE) inhibitors was chemically modified introducing tertiary amine and acyl-amide moieties, and the activities at both ChEs were evaluated. Molecular docking was applied to explain the data and probe the capacity of the mid-gorge site of both ChEs. The novel spacer structures considerably alter the biological profile of bivalent quinazolinimines with regard to both inhibitory activity and selectivity. Mutual interaction of binding to the various sites of the enzymes was further investigated by applying also different spacer lengths and ring sizes of the alicycle of the tricyclic quinazolinimines. In order to achieve selectivity toward BChE and to improve inhibitory activities, the spacer structure was optimized and identified a highly potent and selective BChE inhibitor.

1. Introduction

Two cholinesterases (ChEs[†]), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), regulate cholinergic neurotransmission, the deterioration of which is responsible for the decline in memory and cognition in patients suffering from Alzheimer's disease (AD).[1] Although the decline of cholinergic neurotransmission is most probably a downstream process of AD pathophysiology, until now the only therapeutic intervention uses (apart from the *N*-methyl D-aspartate (NMDA) antagonist memantine) AChE inhibitors for stabilizing memory and cognition decline in AD patients.[2] Approved drugs are in general characterized by comparatively low inhibitory activity and/or a pronounced side-effect profile (e.g., hepatotoxicity of the otherwise potent inhibitor tacrine). In the last couple of years BChE has gained special attention, since its amount in the cortex and hippocampus on post mortem AD brains is up-regulated, whereas the AChE amount is significantly down-regulated.[3] An AChE nullizygous rodent model seems to maintain full CNS functions and BChE seems to be able to compensate for AChE in ACh hydrolysis which is necessary for normal brain function.[4] In general, BChE seems to play a role both in AD pathology and cognition in normal brains.[5] In rats, a BChE selective inhibitor (cymserine) elevated brain ACh levels and augmented long term potentiation and learning.[6] Interestingly, cymserine application also lowered the amount of β-amyloid peptide in the rats' brain.[6] Therefore, either BChE selective compounds or inhibitors with mixed activities might be superior to established AChE inhibitors for improvement of cognitive deficits in more advanced stages of AD.

Several novel strategies have been applied in medicinal chemistry to target AD drug development, for example, well-known inhibitors (like tacrine; Chart 1) have been used in so-called hybrid molecules that covalently connected two distinct drug molecules.[7–10] Additionally, a bivalency approach (formerly mainly applied to GPCR ligands, like opioid agonists and dopamine antagonists [11,12]) has been applied to AChE inhibitors (i.e., to connect covalently two inhibitor molecules by a long hydrocarbon-spacer).[10,13] Most work on AChE inhibitors is based on tacrine and its derivatives.[10,14] Bivalent inhibitors were synthesized with improved inhibitory activities and often increased selectivity toward AChE.[14] But not so much work has been done using the bivalency approach to achieve high inhibitory activity at BChE.[15,16] Recently, we have reported strongly increased BChE inhibition for bivalent compounds derived from tri- and tetracyclic tetahydro-6*H*-isochinolino-[1,2-*a*]isoquinolines and -isoquinolinium salts.[15] Interestingly, BChE selectivity was also achieved with some homo- and especially heterobivalent tacrine-based inhibitors by introducing an alkylated or acetylated nitrogen atom into the spacer.[17,18]

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[†] Abbreviations: AD, Alzheimer's disease; (h)ACh(E), (human) acetylcholine(esterase); BChE, butyrylcholinesterase; BOC, *tert*-butoxycarbonyl; ChE, cholinesterase; DMAP, 4-dimethylaminopyridine; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBt, hydroxybenzotriazole; MOE, molecular operating environment; NMDA, *N*-methyl D-aspartate; SAR, structure–activity relationship.

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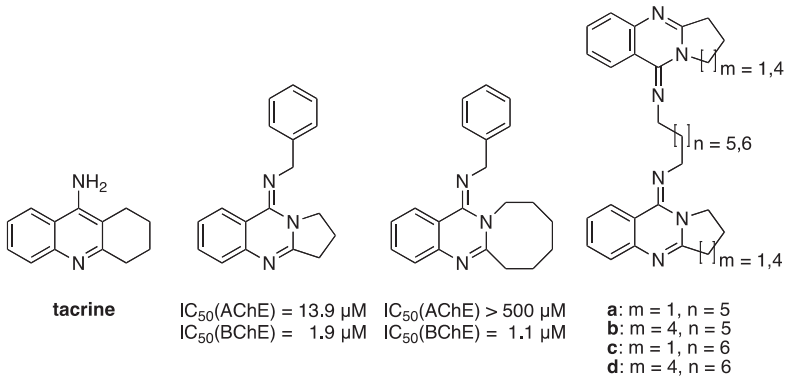
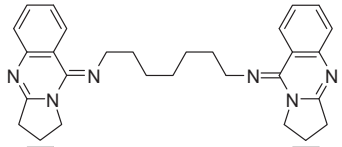
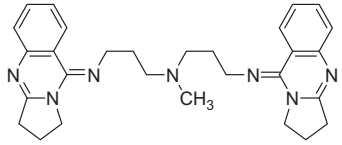
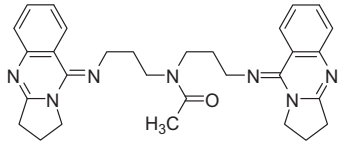
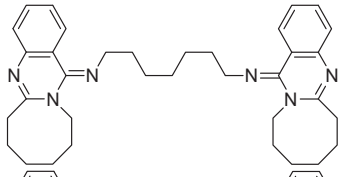
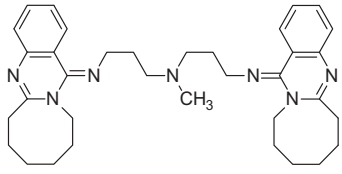


Chart 1. Approved AD drug and ChE inhibitor tacrine; univalent quinazolinimines with the degree of BChE selectivity depending on alicycle size;[19] bivalent quinazolinimines **a**–**d** (see Table 1 for inhibition data).[16]

With regard to the work presented in this paper, we have previously described tricyclic quinazolinimines as a novel class of ChE inhibitors, in which optimization of the alicycle size was used to achieve higher BChE selectivity (Chart 1).[19] Furthermore, bivalent quinazolinimines turned out to be highly potent ChE-inhibitors (with an activity increase of 100-fold compared to the univalent quinazolinimines) (cf. Table 1).[16] Mixed AChE/BChE inhibition was observed for heptamethylene (seven carbon atoms) spacers, irrespective of the ring size of the alicycle (five- to eight-membered rings) (compounds **a** and **b** in the Table 1).[16] By using an octamethylene (eight atoms) spacer an increase in BChE selectivity could be observed with increasing alicycle ring size. Maximum BChE selectivity (almost 190-fold) was achieved with an eight-membered ring system, albeit a concomitant loss in BChE inhibitory activity was observed with increasing alicycle size (compounds **c** and **d** in the Table 1).[16]

Using the results obtained with chemically modified spacers in heterobivalent tacrines and also results obtained with bivalent opioid ligands, in which chemical modifications of the hydrocarbon spacer influenced the binding profile,[20] we decided to incorporate a (tertiary) basic nitrogen atom or an acylated nitrogen atom, respectively, into the spacer structure for both five- and eight-membered ring homobivalent quinazolinimines by applying both seven and eight atom spacers to link the inhibitor parts.[18] Since

Table 1
Inhibition results of target compounds at AChE and BChE and resulting selectivities toward BChE[a]

Compound		IC_{50} (AChE[b]), nM (pIC_{50} ± SEM)	IC_{50} (BChE[c]), nM (pIC_{50} ± SEM)	Selectivity IC_{50} (AChE)/IC_{50} (BChE)
	Galanthamine	640 (6.197 ± 0.052)[19,22]	8400 (5.076 ± 0.033)[19,22]	0.08
	Tacrine	46.5 (7.333 ± 0.037)	6.4 (8.195 ± 0.038)	7.3
a		79 (7.104 ± 0.030)[16]	88 (7.056 ± 0.104)[16]	0.9[16]
17		541.0 (6.267 ± 0.061)	547.1 (6.262 ± 0.066)	1
18		330.7 (6.481 ± 0.038)	267.0 (6.573 ± 0.031)	1.2
b		65 (7.185 ± 0.096)[16]	27 (7.573 ± 0.133)[16]	2.4[16]
19		202.7 (6.693 ± 0.121)	197.0 (6.705 ± 0.089)	1

(*continued on next page*)

Table 1 (*continued*)

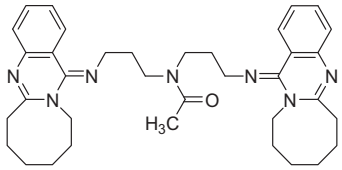
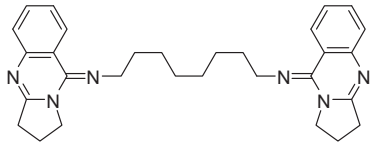
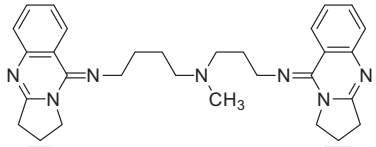
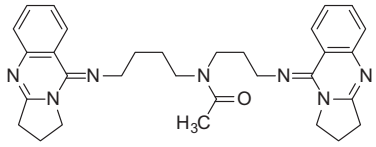
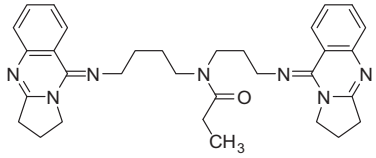
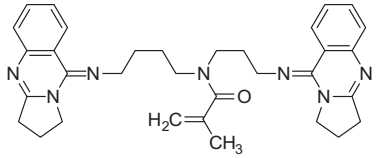
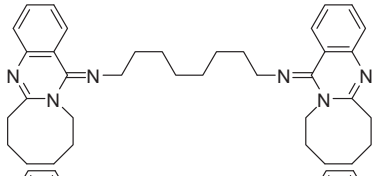
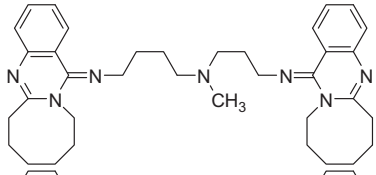
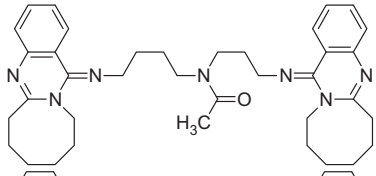
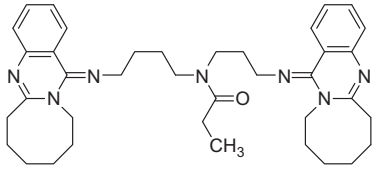
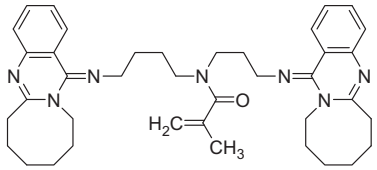
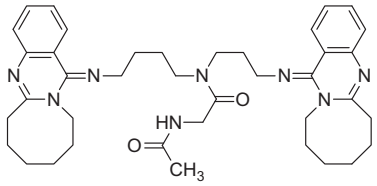
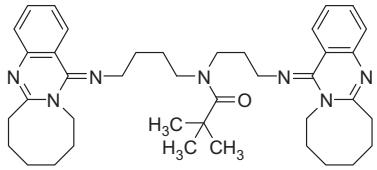
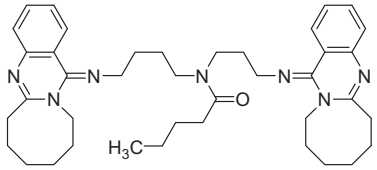
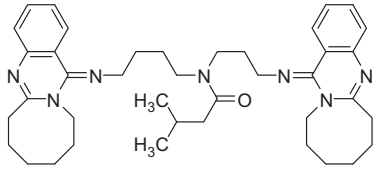
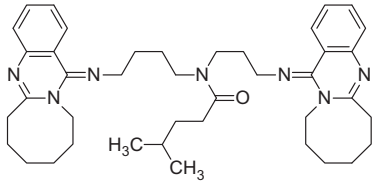
Compound		IC_{50} (AChE[b]), nM (pIC_{50} ± SEM)	IC_{50} (BChE[c]), nM (pIC_{50} ± SEM)	Selectivity IC_{50} (AChE)/IC_{50} (BChE)
20		49.0 (7.310 ± 0.041)	72.7 (7.139 ± 0.034)	0.7
c		58 (7.236 ± 0.054)[16]	4.8 (8.318 ± 0.170)[16]	12[16]
21		560.7 (6.251 ± 0.067)	81.3 (7.090 ± 0.045)	6.9
22		20.9 (7.680 ± 0.051)	4.0 (8.403 ± 0.087)	5
25		18.0 (7.746 ± 0.053)	5.9 (8.230 ± 0.075)	3
27		6.7 (8.174 ± 0.087)	10.6 (7.974 ± 0.090)	0.6
d		14400 (4.842 ± 0.059)[16]	76 (7.120 ± 0.082)[16]	189[16]
23		605.8 (6.218 ± 0.066)	51.4 (7.289 ± 0.033)	11.8
24		177.4 (6.751 ± 0.055)	43.1 (7.365 ± 0.028)	4.2
26		289.0 (6.539 ± 0.089)	46.3 (7.335 ± 0.125)	6.2

Table 1 (*continued*)

Compound		IC_{50} (AChE[b]), nM (pIC_{50} ± SEM)	IC_{50} (BChE[c]), nM (pIC_{50} ± SEM)	Selectivity IC_{50} (AChE)/IC_{50} (BChE)
28		153.2 (6.815 ± 0.163)	37.7 (7.424 ± 0.100)	4.1
29		75.6 (7.122 ± 0.060)	122.1 (6.913 ± 0.149)	0.62
30		536.0 (7.271 ± 0.044)	25.5 (7.593 ± 0.025)	21
31		1056.0 (5.976 ± 0.109)	12.9 (7.889 ± 0.037)	81.9
32		621.0 (6.207 ± 0.056)	8.1 (8.093 ± 0.084)	76.7
33		792.1 (6.101 ± 0.024)	3.0 (8.525 ± 0.019)	264

[a] Values are means of at least three independent determinations, $pIC_{50} = -\log IC_{50}$.
[b] EC 3.1.1.7, type VI-S, from Electric Eel.
[c] EC 3.1.1.8, from equine serum.

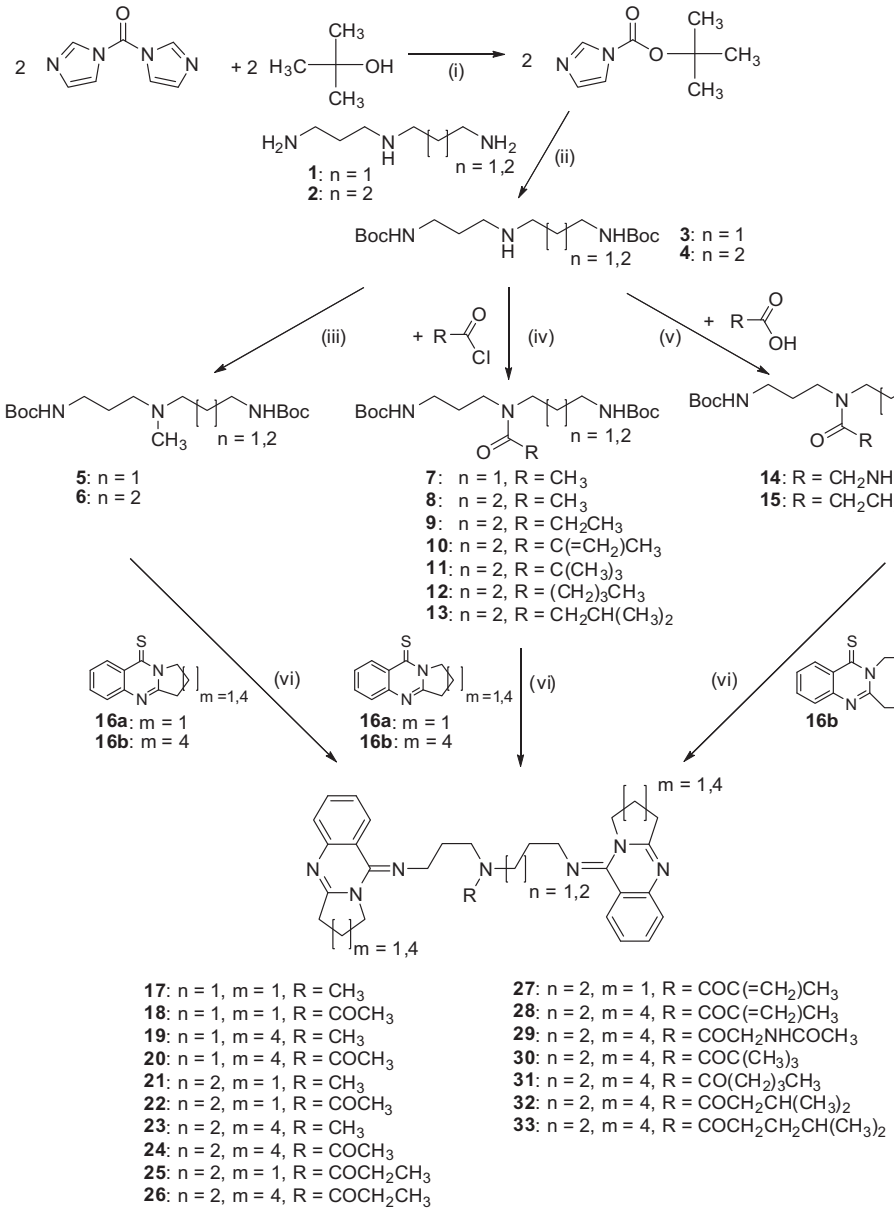
the bivalent quinazolinimines had previously been optimized in terms of heterocycle structure,[19,21] the size of the alicycle,[19,22] and the spacer length,[16] respectively, the spacer's chemical structure remained to be optimized in this set of structure–activity relationships (SARs) using knowledge about the enzymes' binding sites.

In this work we have applied molecular docking parallel to the syntheses to understand the binding profiles of available inhibitors that we synthesized and to predict novel structures with optimal spacer interaction with both ChEs or with selectivity towards BChE. To probe the mid-gorge binding sites of the ChEs involving interaction with the spacer substructures, we synthesized a set of compounds in which a central nitrogen atom in the spacer was modified as a basic *N*-CH_3 group or various *N*-acylated groups. To probe successively the mid-gorge binding site (while using both five- and eight-membered alicycle size and seven and eight atom spacers) we incorporated *N*-acyl groups containing H-bond acceptors, donors, as well as hydrophobic moieties. The preference of hydrophobic substitutions to increase inhibitory activity of the compounds was further investigated in terms of their length and steric expansion to yield optimal activity and selectivity toward BChE.

2. Chemistry

The target spacer-modified bivalent quinazolinimines (**17**–**33**) were synthesized from quinazolinethiones (**16a**,**b**) and appropriate α,ω-diamines (**5**–**15**) (Scheme 1). The heterocyclic key intermediate five- and eight-membered ring quinazolinethiones (2,3-dihydropyrrolo[2,1-*b*]quinazoline-9(1*H*)-thione **16a** and 8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazoline-13(7*H*)-thione **16b**) were synthesized from the respective five-membered ring lactam (pyrrolidin-2-one) and eight-membered ring lactam (azocan-2-one) by reaction with Meerwein salt (triethyloxonium tetrafluoroborate) to yield the reactive iminium ethers, which reacted with anthranilic acid to yield the respective tricyclic five-membered (2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-one) and eight-membered ring quinazolinones (8,9,10,11-tetrahydro-6H-azocino [2,1-*b*]quinazolin-13(7*H*)-one).[19,23] The oxygen-atom of the quinazolinones could be replaced by a sulphur atom using Lawesson's reagent to yield compounds **16a**,**b**.[16,19]

For selective alkylation (**5** and **6**) and acylation (**7**–**15**) of the middle secondary amine-group of dipropylenetriamine (**1**) and



Scheme 1. Synthesis of spacer-modified bivalent quinazolinimines **17**–**33** with varying spacer lengths and modified chemical structures of the spacer. Reagents and conditions: (i) KOH, toluene, 120 °C, 4 h; (ii) dipropylenetriamine (**1**) or spermidine (**2**), 60 °C, 4 h; (iii) CH_3I, K_2CO_3, CH_3CN, rt, 24 h; (iv) NEt_3, cat. DMAP, CH_2Cl_2, 5 °C→rt, 3 h; (v) EDCI, HOBt, CH_2Cl_2, rt, 12 h; (vi) (1) 4 M HCl in 1,4-dioxane, CH_2Cl_2, rt, 12 h; (2) $HgBr_2$, Na_2CO_3, EtOH/toluene, 135 °C, 2 h.

spermidine (**2**), respectively, selective protection of both primary amino-groups was necessary, which was achieved using *N*,*N*′-carbonyldiimidazole, *tert*-butanol and KOH in toluene to yield the di-Boc-protected compounds with a free secondary amine function (**3** and **4**; Scheme 1).[24] Methylation of di-Boc-protected dipropylenetriamine (**5**) and spermidine (**6**) was achieved by methyl iodide and potassium carbonate in acetonitrile, whereas acylation was achieved using the respective acid chlorides, triethylamine and catalytic amounts of DMAP (Scheme 1). Alternatively, acylation of **4** could be achieved using the free carbonic acid, EDCI and catalytic amounts of HOBt (**14** and **15**, Scheme 1).

For the formation of bivalent quinazolinimines (**17**–**33**) we used N-methylated and N-acylated α,ω-diamines as starting materials for the spacer structure in the form of their hydrochlorides, which were obtained from the di-Boc-protected forms (**5**–**15**) using 4 M HCl in 1,4-dioxane. The method for condensation with quinazolinethiones had to be slightly modified compared to previously applied method[16,19] due to altered solubility properties of the starting materials and the use of the spacer hydrochlorides: A 1/1 mixture of toluene and ethanol was used as solvent, sodium carbonate and mercury(II) bromide as reagents (Scheme 1).

3. Results and discussion

We not only wanted to gain information on whether and how the binding and inhibition profile of bivalent quinazolinimines is influenced by the spacer structure, but also to investigate if the spacer structure alone, or if the length of the spacer and the size of the alicycle concomitantly modify the inhibition profile. Therefore, five- and eight-membered ring systems, in which five-membered alicycles in tricyclic quinazolinimines exhibit the highest activity at AChE in the set of alkylene bridged bivalent compounds,[16] were investigated and spacer lengths of seven and eight atoms were synthesized and their inhibition profiles investigated systematically in correlation to different spacer structures. Enzyme inhibition was determined applying the colorimetric Ellman assay

using AChE (EC 3.1.1.7, type VI-S, from electric Eel) and BChE (E.C. 3.1.1.8, from equine serum), with acetylthiocholine (ATC) and butyrylthiocholine (BTC) respectively as substrates.[19,21]

Altogether, seventeen novel bivalent inhibitors were synthesized. In the set of seven atom spacer bridged compounds (**17**–**20**), activities were generally lower than for the alkylene-bridged parent compounds (**a**, **b**), with both *N*-CH_3 (**17** and **19**) and *N*-$COCH_3$ (**18** and **20**) groups incorporated into the spacer (inhibitory activities ranged from IC_{50} = 49 nM for **20** to 541 nM for **17** at AChE). Especially the *N*-CH_3 groups led to a three to sevenfold (**17**) loss in activity at AChE and at BChE as well. As for the parent compound, activity was almost identical at both ChEs. The introduction of an acetyl group into the spacer of heterobivalent tacrines was described in the literature to improve BChE selectivity suggesting that the mid-gorge interaction site might be a promising binding spot to shift selectivity towards BChE.[17] For the *N*-$COCH_3$ compounds in the set of bivalent quinazolinimines, loss in activity was observed, but it was far less pronounced than for the *N*-CH_3 compounds (with the only positive exception of a slightly increased activity at AChE for the eight-membered ring bivalent compound **20**, IC_{50}(AChE) = 49 nM and IC_{50}(BChE) = 73 nM).

The binding data was different for quinazolinimines with eight atoms spacers. And as for the seven atom bridged quinazolinimines, their binding data was also completely different from the data of bivalent tacrines.[17,18] For five-membered ring quinazolinimines, *N*-CH_3 led to a dramatic activity decrease at both ChEs (**21**, IC_{50}(AChE) = 561 nM and IC_{50}(BChE) = 81 nM).[7] For the eight-membered ring quinazolinimines there was a slight increase in BChE activity, and surprisingly a 24-fold activity increase at AChE also, which reduced the selectivity from 189-fold to 12-fold (**23**). An *N*-$COCH_3$ group led to activity increase (**22** and **24**) compared to the alkylene parent compound: The high activity at BChE was very slightly enhanced with both five- and eight-membered alicycle quinazolinimines, activity at AChE was improved threefold for five-membered rings (**22**, IC_{50}(AChE) = 21 nM and IC_{50}(BChE) = 4 nM) and 81-fold for eight-membered rings (**24**, IC_{50}(AChE) = 177 nM and IC_{50}(BChE) = 43 nM). Notably, inhibition data at both AChE and BChE was therefore considerably different from the data of the homobivalent compounds connected by a hydrocarbon spacer.

We then investigated computationally the binding of *N*-acetylated bivalent compounds **22** (five-membered ring alicycles) and **24** (eight-membered ring alicycles) with AChE and BChE to provide structural insights and potential to functional modifications. For that we constructed the homology models of the investigated species, that is, equine BChE and electric eel AChE, using the crystal structure of Torpedo californica AChE in the complex with a bivalent inhibitor (PDB code: 2CEK),[25] thus we generated a bivalent inhibitor-adapted conformation to simplify docking studies. We built the 3D models of the enzyme species used for our biological experiment for docking studies, as it has shown that the close series of analogs binds to enzyme species differently,[17,26,27] despite the high sequence homology among species (more then 80%).[28] Thus, we noted that V305, L313 and I426 of the binding site (7Å from the ligand) in the equine species of BChE are substituted to corresponding ALA, PRO and PHE in human BChE. In analogy S98, F309, S310, G311 in the electric eel AChE located at the entrance to the binding cavity correspond to LEU, GLN, GLU, SER in human AChE. We hypothesize that these residues might be important for species selectivity. The structural models of AChE and BChE in cartoon representation coloured based on the secondary structure and the ligand-binding site shown in the form of the Connolly surface with docked **22** (i.e., acetylated eight atoms spacer compounds with five-membered ring systems) in the space filling representation are shown in Figure 1A (BChE) and B (AChE). The ligand-binding cavity of BChE (Fig. 1A) is more spacious compared to the ligand-binding cavity of AChE (Fig. 1B). The extra-volume in BChE is gained due to non-conserved residues, which exhibit different physicochemical and geometrical properties compared to the properties of residues in AChE. Thus, aromatic residues N96, Q147, V305, L314, A356 lining the binding cavity of BChE are substituted by Y94, Y146, W304, F315, Y355 correspondently in AChE. The large volume of the BChE binding cavity could explain the higher binding tolerance to the structural modifications of the compounds.

The detailed pictures of the superimposed docking poses of **22** and **24** are also shown in Figure 1 (C for BChE and D for AChE respectively). As expected, the heterocyclic parts of bivalent quinazolinimine inhibitors form characteristic aromatic interactions in these enzymes. Thus, in BChE, one aromatic moiety is engaged in π-π stacking interactions with W110 of BChE, whilst the second moiety locates around V305, D311 and F306 according to our docking studies.

In AChE, the bivalent inhibitor has two π-π stacking interactions between one quinazolinimine moiety and W108, and another quinazolinimine moiety and W304 and Y94. The acyl group of the linker forms hydrogen bond with Q147 in BChE and Y146 in AChE, that provides a new favourable interaction with the enzyme. We used the Site Map of Schrödinger software to predict the preferable regions for hydrophobic and hydrophilic interactions, which are also shown in Figure 1C and D. Accordingly, the regions with the preference of hydrophobic groups are represented in yellow, while the regions with the preference of acceptor and donor groups are shown in red and blue, respectively. The Site Map shows that there is possibility to increase the size of the functional group of the spacer using hydrophobic substituents in order to increase potency of inhibition especially toward BChE. This information was applied in the next synthetic sequence.

In the case of *N*-acetyl substituent compounds, the remarkable increase in AChE activity together with the slight increase at BChE activity actually led to decreased selectivity. Given that there are preferable regions around the mid-gorge of BChE we wanted to increase this selectivity while maintaining high BChE activity by taking advantage of the mid-gorge region. For this reason the acyl moiety of bulky eight-membered quinazolinimines was further modified, whereas *N*-alkyl substituents were not investigated further because of their poor biological profile in relation to potency and BChE selectivity. A similar observation was later also made for a different class of AChE hybrid inhibitors described by Pisani et al.[7] Apart from the available non-conserved residues in the mid-gorge regions of the ChE binding site, which could be targeted to design selective ligands, the available crystal structures of ChEs as well as molecular dynamics studies of unoccupied and occupied states of the enzymes have shown that there is a high degree of enzyme flexibility during the binding of an allosteric ligand that causes substantial structural changes in the catalytic site.[29–32] Importantly, the bioinformatics analysis of available crystal structures shows that the residues in the mid-gorge are likely in cooperative motions.[18] Intuitively, the blockade of their mobility could lead to modulation of enzyme inhibition. We therefore persisted in modification of the mid-gorge spacer to modulate enzyme selectivity and improve potency.

We replaced the acetyl by a propionyl group (**26** with eight-membered alicycles, **25** with five-membered alicycles) and found that the propionyl showed the same binding profile as acetyl at both ChEs, with a slightly increased BChE selectivity for **26**. Methylacryloyl substituents (**27** and **28**), which should be able to be polarized and thus interact with polar groups of the mid-gorge predicted by Site Map, led to an around twofold increase in AChE activity compared to the propionyl compounds (both for five- and eight-membered compounds) without significantly affecting BChE activity. As a result, five-membered rings were abandoned

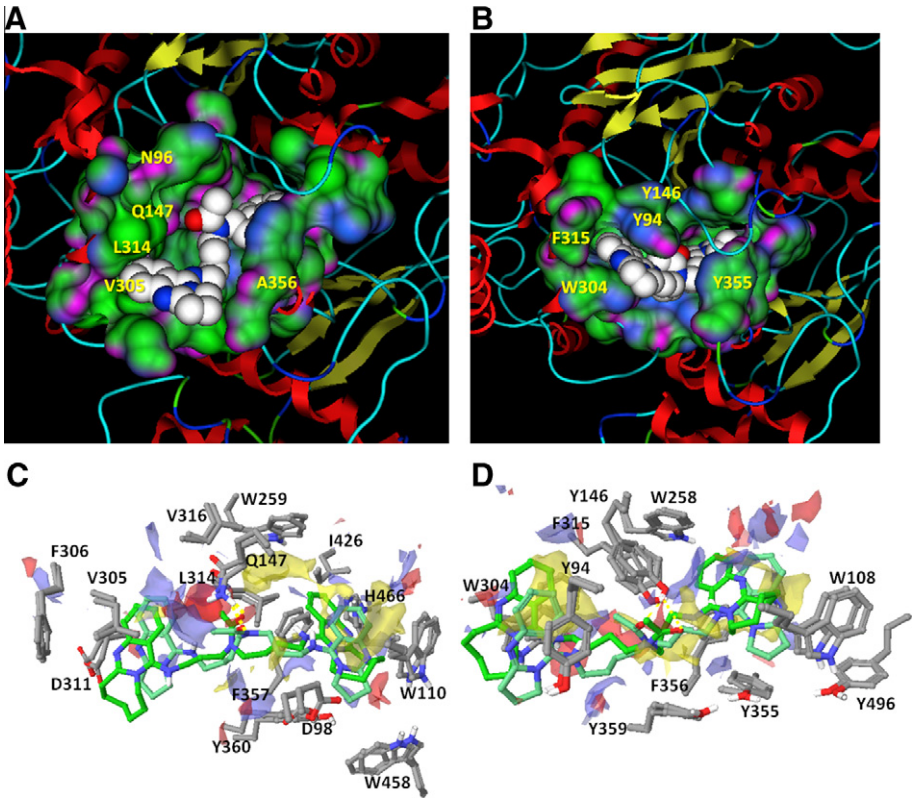


Figure 1. The binding site of spacer-modified (*N*-acetylated) bivalent quinazolinimines **22** and **24** in butyrylcholinesterase (BChE) and acetylcholinesterase (AChE). (A and B)—the ligand-binding cavity of BChE (A) and AChE (B) in the Connolly surface representation coloured based on the hydrophilic and hydrophobic properties (hydrophobic regions are in green, receptor donor regions are in purple and receptor acceptor regions are in blue) with docked compound **22** having a eight atoms spacer and the *N*-$COCH_3$ group in the space filling representation. The enzyme structure is shown in cartoons and coloured based on the secondary structure. The non-conserved residues are labelled in yellow. (C and D)—the superimposed binding site of compounds **22** and **24** in BChE (C) and in AChE (D). The ligands are shown in stick-like representation with the carbon atoms coloured in dark and light green. The residues of the binding sites are in stick-like representation with the carbon atoms coloured in grey. The preferable regions for hydrophobic and hydrophilic interactions predicted by the Site Map (Schrödinger software) are shown in yellow (hydrophobic group preference), red (acceptor group preference) and blue (donor group preference).

at this stage because they represented no promising contribution to the improvement of the potency and selectivity focusing on BChE. Yet the potential interaction of the substituent with polar groups in the gorge led to the incorporation of an *N*-acetylglycine moiety (**29**), which contains both H-bond-accepting and -donating groups and might exhibit the interaction with other amino acids within the mid-gorge of the enzymes. Surprisingly, despite its long and sterically demanding spacer group, **29** shows IC_{50} values of 122 nM at BChE and 76 nM at AChE (Table 1). In contrast to previous results obtained with bis-tacrines, this sterically demanding moiety is obviously able to interact well with AChE resulting in low IC_{50} value at AChE. To explain this unexpected biological result, **29** was docked to the binding sites of both ChEs (Fig. 2A and B). Our docking studies on the *N*-acetylglycine compound **29** (BChE in Fig. 2A and AChE in B) show that the acetylglycine substituent occupies a different position in the enzymes, thus it faces Q147 forming hydrogen bonds with its side chain and the backbone of L314 in BChE (Fig. 2A), whilst it orientates in somewhat the opposite direction forming a hydrogen bond with the backbones of F313 in AChE (Fig. 2B). The docking score of compound **29** is −17 kcal/mol in AChE whereas −11.4 kcal/mol in BChE, indicating the preference in binding to AChE. This is in agreement with the predicted hydrophilic binding sites by Site Map. Different location of the acetylglycine group might account for the observed comparatively high potency in both ChEs. Since our search was aiming to design BChE-selective compounds, the comparatively low IC_{50} value at BChE and the availability of preferable hydrophobic zones calculated by Site Map encouraged us to probe sterically even more demanding moieties into the mid-gorge that lack the possibility to interact with polar groups.

A sterically demanding 2,2-dimethylpropanoyl moiety (**30**) showed improved activity at BChE (compared to methylacryloyl and propionyl) with an IC_{50}(BChE) of 26 nM. But despite the three-fold increase in BChE activity compared to the alkylene-bridged parent compound (**d**), the compound's 21-fold BChE selectivity is still lower. An interesting development was observed with even longer chains: an unbranched pentanoyl moiety attached to the spacer (**31**) showed 82-fold selectivity with an IC_{50}(BChE) of 13 nM followed by a shorter 3-methylbutanoyl moiety (**32**) that caused a further activity increase to an IC_{50}(BChE) of 8 nM with similar 77-fold selectivity. In the end, the combination of the longer chain with additional methyl-substitution to 4-methylpentanoyl-(**33**) led to the most potent and selective compound in the whole series of compounds with an IC_{50}(BChE) of 3 nM and 260-fold selectivity over AChE (IC_{50} = 792 nM). Compound **33** therefore surpasses the most selective compound in the alkylene-bridged series of bivalent quinazolinimines (**d**) being 25-fold more active at BChE with even improved selectivity. Even the most potent, but unselective alkylene-bridged bivalent quinazolinimine with five-membered ring alicycles (**c**) could be surpassed in BChE activity with **33**.

In order to explain the molecular basis of high potency and selectivity of **33** to BChE we have docked this compound to BChE (Fig. 2C) and AChE binding sites as well (Fig. 2D). The most

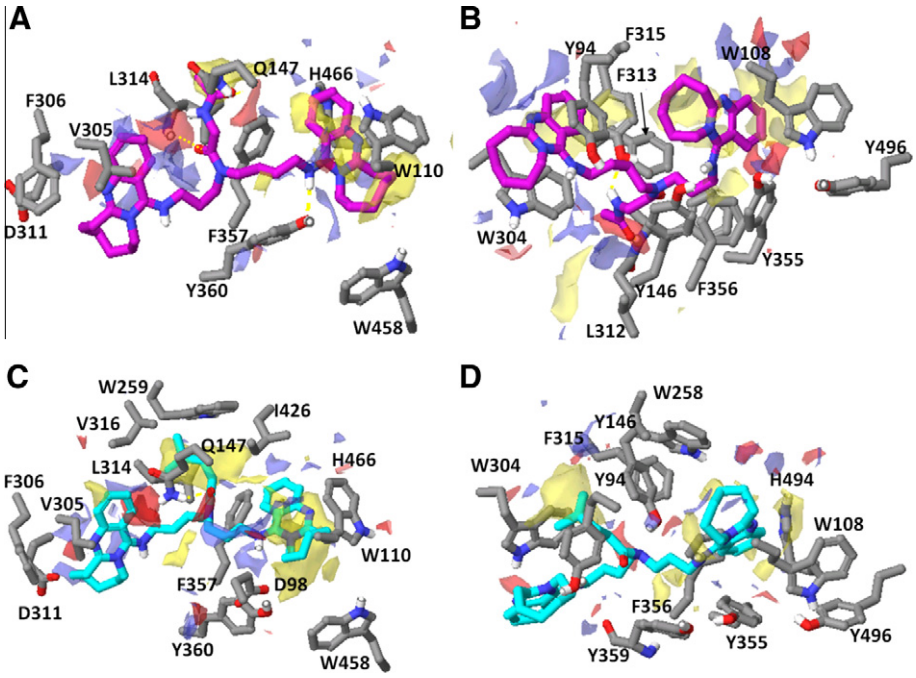


Figure 2. The preferable binding pose of compounds **29** and **33** in butyrylcholinesterase (BChE), (A and C) and acetylcholinesterase (AChE), (B and D) obtained by Induced Fit docking (Schrödinger software). The ligands and the binding sites are in stick-like representation, compound **29** is in purple, compound **33** is in cyan and the residues of the binding site are in grey. The prefarable regions for hydrophobic and hydrophilic interactions predicted by the Site Map (Schrödinger software) are shown in yellow (hydrophobic group preference), red (acceptor group preference) and blue (donor group preference).

populated docking poses are shown in Figure 2C and D. Thus, in BChE the functional group of the spacer locates in the hydrophobic binding pocket composed of L314, V316 and W259 which is shown in yellow in Figure 2C. This preferable interaction leads to an increase of compound affinity. Since L314 and V316 in BChE correspond to F313 and F315 in AChE, the new hydrophobic interaction may be responsible for selectivity of **33**. On the contrary, there is no empty pocket with preference to hydrophobic substituents in AChE because the corresponding F313 and F315 are involved into the intramolecular aromatic interaction network. However, there is the hydrophobic pocket involving Y94, W304 and F315 mapped in yellow on Figure 2D. Indeed, our docking studies show that the preferable pose for the aliphatic substituent of **33** is in the region of these residues in AChE. However, this interaction in AChE partially fills the space between W304 and F315 and thus prevents the formation of the π-π electron sandwich with one quinazolinimine, one of the key interactions of these bivalent inhibitors with ChEs. The loss of this interaction likely leads to the significant loss of potency of **33** in AChE. Accordingly, the quantitative docking score of **33** in the chosen poses is −9 kcal/mol in AChE and −10.5 kcal/mol in BChE supporting high affinity of **33** to bind BChE. But in general, it turned out to be hard to get a reliable prediction of experimental inhibitory activity from the docking procedures performed due to interactions of the spacer moieties with the enzymes' structure that impede reliable predictions.

4. Conclusion

Novel bivalent quinazolinimines were synthesized in which a nitrogen atom was introduced into the alkylene hydrocarbon spacers to yield tertiary amines or *N*-acyl amides. Apart from the spacer structure the alicycle ring size of the heterocyclic quinazolinimines and the spacer length were also modified to investigate mutual interactions on compound activity and selectivity. Chemical modification of the spacer structure shows great influence on the binding profile in terms of activity and especially selectivity. Intensive investigation into the different structural parameters identified compound **33** (showing an eight atom spacer, eight-membered ring alicycles and a 4-methylpentanoyl moiety as the acyl part) as a highly potent and selective inhibitor of BChE. Molecular docking of *N*-acetylglycine derivative (**29**) incorporating H-bond-accepting and -donating properties was performed and yielded low energy differences, which on the contrary suggested that the hydrophobic interaction of the 4-methylpentanoyl moiety with the mid-gorge site of the enzyme is responsible for high potency of compound **33**. Taken together, the different shape and physicochemical properties of the mid-gorge site of cholinesterases as well as its flexibility provide the possibility to modify the spacer of bivalent ligands leading to novel binding profiles with high BChE potency and selectivity.

5. Experimental section

5.1. Chemistry

Melting points are uncorrected and were measured in open capillary tubes, using a Barnstead Electrothermal IA9100 melting point apparatus. ^{1}H and ^{13}C NMR spectral data were obtained from a Bruker Advance spectrometer (400 MHz and 125 MHz, respectively). TLC was performed on silica gel on aluminium foils with fluorescent indicator 254 nm (Fluka) or aluminium oxide on TLC-PET foils with fluorescent indicator 254 nm (Fluka). For detection iodine vapour or UV light (254 nm), respectively, were used. ESI-MS samples were analyzed using electrospray ionisation ion-trap mass spectrometry in nanospray mode using a Thermo Finnigan LCQ Deca. The CHN analyses were undertaken using Perkin Elmer Elemental Analyser PE2400CHNS. For column chromatography, silica gel 60, 230–400 mesh (Merck) or activated neutral aluminium oxide, approximately 150 mesh, 58Å (Sigma–Aldrich) were used.

5.1.1. *N*-(*t*-Butoxycarbonyl)-*N*′-[3-(*t*-butoxycarbonylamino)-propyl]-1,3-propanediamine (3)

Compound **3** was synthesized according to the method described in Ref. 24. Colourless oil, yield 66 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37 (s, 18H, 2 × Boc), 1.56 (m, 4H, 2 × $NHCH_2CH_2$), 2.58 (t, 4H, J = 3.0 Hz, 2 × $NHCH_2$), 3.14 (t, 4H, J = 6.0 Hz, 2 × $BocNHCH_2$), 5.15 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 27.43 ($C(CH_3)_3$), 28.79 ($NHCH_2CH_2$), 37.96 ($BocNHCH_2$), 46.43 ($NHCH_2$), 79.28 ($NHCOOC(CH_3)_3$), 155.12 (NH*C*OO*t*Bu) ppm. Spectral data is in accordance with literature data.[24]

5.1.2. *N*-(*t*-Butoxycarbonyl)-*N*′-[3-(*t*-butoxycarbonylamino)-propyl]-1,4-butanediamine (4)

Compound **4** was synthesized according to the method described in Ref. 28. Colourless oil, yield 61 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.36–1.45 (m, 22H, 2 × Boc, $NHCH_2(CH_2)_2CH_2NH$), 1.57 (m, 2H, $NHCH_2CH_2CH_2NH$), 2.53 (t, 2H, J = 6.64 Hz, $NHCH_2(CH_2)_3NHBoc$), 2.58 (t, 2H, J = 6.61 Hz, $NHCH_2(CH_2)_2NHBoc$), 3.05 (m, 2H, $BocNHCH_2$), 3.12 (m, 2H, $BocNHCH_2$), 4.99 (br s, 1H, N*H*Boc), 5.28 (br s, 1H, N*H*Boc) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 27.69, 28.16, 28.78, 30.21, 39.50, 40.76, 48.00, 49.76, 79.16 ($OC(CH_3)_3$), 156.45 (NH*C*OO*t*Bu) ppm. Spectral data is in accordance with literature data.[28]

5.1.3. *N*-(*t*-Butoxycarbonyl)-*N*′-[3-(*t*-butoxycarbonylamino)-propyl]-*N*′-methyl-1,4-butanediamine (6)

Compound **6** was synthesized according to the method described in Ref. 18. Viscous yellow liquid, yield 30 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37 (s, 18H, 2 × Boc), 1.44 (m, 4H, $NHCH_2(CH_2)_2CH_2NH$), 1.59 (m, 2H, $NHCH_2CH_2CH_2NH$), 2.13 (s, 3H, NCH_3), 2.28–2.35 (m, 4H, 2 × $CH_2N(CH_3)$), 3.05–3.12 (m, 4H, 2 × CH_2NHBoc), 4.87 (s, 1H, N*H*), 5.32 (s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 24.90, 27.22, 28.19, 28.88 ($C(CH_3)_3$), 40.22 ($NHCH_2$), 40.80($NHCH_2$), 42.15 (NCH_3), 56.66 (NCH_2), 57.69 (NCH_2), 79.30 ($OC(CH_3)_3$), 79.37 ($OC(CH_3)_3$), 156.48 (NH*C*OO*t*Bu), 156.52 (NH*C*OO*t*Bu) ppm. Spectral data is in accordance with literature data.[18]

5.1.4. *N*,*N*-Bis[3-(*t*-butoxycarbonylamino)propyl]acetamide (7)

Compound **7** was synthesized according to the method described in Ref. [18]. Viscous yellow liquid, yield 100 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37 (d, 18H, 2 × Boc), 1.57–1.62 (m, 2H, $NHCH_2CH_2$), 1.68–1.75 (m, 2H, $NHCH_2CH_2$), 2.02 (s, 3H, $NCOCH_3$), 3.00 (q, 2H, J = 6.14 Hz, NCH_2), 3.08 (q, 2H, J = 6.33 Hz, NCH_2), 3.20 (t, 2H, J = 7.78 Hz, $NHCH_2$), 3.32 (t, 2H, J = 6.46 Hz, $NHCH_2$), 4.62 (br s, 1H, N*H*), 5.29 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 21.79 ($COCH_3$), 28.36, 28.80, 28.88, 30.09, 37.66, 38.53, 42.81, 46.63, 79.32 ($COOC(CH_3)_3$), 80.00 ($COOC(CH_3)_3$), 156.42 (NH*C*OO*t*Bu), 156.57 (NH*C*OO*t*Bu), 171.22 (*C*OCH_3) ppm. Spectral data is in accordance with literature data.[18]

5.1.5. *N*-Acetyl-*N*′-(*t*-butoxycarbonyl)-*N*-[3-(*t*-butoxycarbonylamino)propyl]-1,4-butanediamine (8)

Compound **8** was synthesized according to the method described in Ref. 18. Viscous yellow liquid, yield 80 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.46–1.60 (m, 24H, 2 × Boc, $NHCH_2(CH_2)_2CH_2NH$, $NHCH_2CH_2CH_2NH$), 2.09 (s, 3H, $NCOCH_3$), 3.08–3.41 (m, 8H, 2 × NCH_2, 2 × $NHCH_2$), 4.57 (br s, 1H, N*H*), 5.32 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 18.56 ($COCH_3$), 25.65, 26.17, 27.22, 27.58, 27.91, 28.47, 28.53, 37.24, 39.76, 43.40, 45.52, 78.68 ($OC(CH_3)_3$), 79.15 ($OC(CH_3)_3$), 156.42 (NH*C*OO*t*Bu), 172.54 ($NCOCH_3$) ppm. ESI-MS m/z 388.3 $[M+H]^+$. Spectral data is in accordance with literature data.[18]

5.1.6. *N*-Propionyl-*N*′-(*t*-butoxycarbonyl)-*N*-[3-(*t*-butoxycarbonylamino)propyl]-1,4-butanediamine (9)

To a solution of compound **4** (1.4 g, 4 mmol), triethylamine (0.67 mL, 4.8 mmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane (10 mL) was added dropwise a solution of propionyl chloride (0.67 mL, 4.4 mmol) in dichloromethane (10 mL) at 5 °C. After the addition, the solution was stirred at room temperature for 3 h. To the mixture 5% citric acid aqueous solution (15 mL) was added and the aqueous layer was separated. The organic layer was dried over $MgSO_4$, filtered and solvent evaporated. The crude product was purified by column chromatography (dichloromethane/methanol, 20:1) to yield the product (1.27 g, 79 %) as a viscous yellow liquid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.07 (t, 3H, J = 7.42 Hz, $COCH_2CH_3$), 1.35–1.70 (m, 24H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$), 2.26 (q, 2H, J = 7.46 Hz, $NCOCH_2$), 2.96–3.34 (m, 8H, 2 × NCH_2, 2 × $BocNHCH_2$), 4.69 (br s, 1H, N*H*Boc), 5.41 (br s, 1H, N*H*Boc) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 9.71 ($COCH_2CH_3$), 24.95, 25.97, 26.22, 27.58, 27.91, 28.38, 28.44, 37.17, 39.85, 42.32, 45.40, 78.79 ($OC(CH_3)_3$), 79.26 ($OC(CH_3)_3$), 156.05 (NH*C*OO*t*Bu), 173.38 ($NCOCH_2CH_3$), 174.18 ($NCOCH_2CH_3$) ppm. ESI-MS m/z 402.3 $[M+H]^+$.

5.1.7. *N*-Methacryloyl-*N*′-(*t*-butoxycarbonyl)-*N*-[3-(*t*-butoxycarbonylamino)propyl]-1,4-butanediamine (10)

This compound was prepared analogously to compound **9**. Compound **4** (1.5 g, 4.35 mmol), triethylamine (0.73 mL, 5.25 mmol) and methacryloyl chloride (0.467 mL, 4.78 mmol) were used and the crude product was purified by column chromatography (dichloromethane/methanol, 20:1 as eluent) to yield the product (1.6 g, 89 %) as a viscous colourless liquid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.36–1.52 (m, 22H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$), 1.64–1.65 (m, 2H, $NCH_2CH_2CH_2NH$), 1.88 (s, 3H, $COC(CH_3)=CH_2$), 3.00–3.04 (m, 4H, 2 × NCH_2), 3.24–3.39 (m, 4H, 2 × $BocNHCH_2$), 4.70 (br s, 1H, N*H*), 4.92 (s, 1H, =C*H*), 5.06 (s, 1H, =C*H*), 5.40 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 20.65 ($COC(CH_3)=CH_2$), 26.14, 27.39, 27.79, 28.37, 28.39, 37.31, 39.95, 41.05, 48.11, 78.84 ($OC(CH_3)_3$), 79.14 ($OC(CH_3)_3$), 114.55 ($=CH_2$), 141.00 ($COC(CH_3)=CH_2$), 156.01 (NH*C*O-O*t*Bu), 173.19 (*C*$OC(CH_3)=CH_2$) ppm. ESI-MS m/z 414.4 $[M+H]^+$.

5.1.8. *N*-[4-(*t*-Butoxycarbonylamino)butyl]-*N*-[3-(*t*-butoxycarbonylamino)propyl]pivalamide (11)

This compound was prepared analogously to compound **9**. Compound **4** (0.5 g, 1.45 mmol), triethylamine (0.24 mL, 1.74 mmol) and trimethylacetyl chloride (0.19 mL, 1.5 mmol) were used and the crude product was purified by column chromatography (dichloromethane:methanol, 20:1 as eluent) to yield the product (0.5 g, 80 %) as a colourless oil.^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.19 (s, 9H, $NHCOC(CH_3)_3$), 1.37–1.66 (m, 24H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$), 3.00–3.07 (m, 4H, 2 × NCH_2), 3.25–3.33 (m, 4H, 2 × $BocNHCH_2$), 4.82 (br s, 1H, N*H*), 5.21 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 26.44, 27.12, 27.38, 28.35, 28.37, 28.47, 38.04, 38.32, 39.00, 40.14, 40.24, 50.32, 79.27 ($OC(CH_3)_3$), 79.37 ($OC(CH_3)_3$), 156.27 (NH*C*OO*t*Bu), 156.32 (NH*C*OO*t*Bu), 177.64 ($NCOC(CH_3)_3$) ppm. ESI-MS m/z 430.2 $[M+H]^+$.

5.1.9. *N*-[4-(*t*-Butoxycarbonylamino)butyl]-*N*-[3-(*t*-butoxycarbonylamino)propyl] pentanamide (12)

This compound was prepared analogously to compound **9**. Compound **4** (0.5 g, 1.45 mmol), triethylamine (0.24 mL, 1.74 mmol) and *n*-pentanoyl chloride (0.182 mL, 1.5 mmol) were used and the crude product was purified by column chromatography (dichloromethane:methanol, 20:1 as eluent) to yield the product (0.51 g, 82 %) as a colourless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.85 (t, J = 7.32 Hz, CH_3), 1.18–1.72 (m, 28H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$, $COCH_2(CH_2)_2CH_3$), 2.22 (q, 2H, J = 7.60 Hz, $COCH_2$), 2.95–3.34 (m, 8H, 2 × NCH_2, 2 × $BocNHCH_2$), 4.62 (br s, 1H, N*H*), 5.40 (br s, 1H, N*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 13.87 (CH_3), 22.56 (CH_2CH_3), 27.61, 27.67, 28.38, 28.44, 32.80 ($COCH_2$), 42.45, 45.40, 45.57, 47.47, 53.38, 78.87 ($OC(CH_3)_3$), 79.35 ($OC(CH_3)_3$), 156.04 (NH*C*OO*t*Bu), 172.90 ($NCOCH_2$), 173.60 ($NCOCH_2$) ppm. ESI-MS m/z 430.2 $[M+H]^+$.

5.1.10. 3-Methyl-*N*-[4-(*t*-butoxycarbonylamino)butyl]-*N*-[3-(*t*-butoxycarbonylamino)propyl] butanamide (13)

This compound was prepared analogously to compound **9**. Compound **4** (0.5 g, 1.45 mmol), triethylamine (0.24 mL, 1.74 mmol) and 3-methylbutyryl chloride (0.182 mL, 1.5 mmol) were used and the crude product was purified by column chromatography (dichloromethane:methanol, 20:1 as eluent) to yield the product (0.5 g, 80 %) as a colourless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.87–0.89 (m, 6H, $CH(CH_3)_2$), 1.31–1.71 (m, 25H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$, $CH(CH_3)_2$), 2.96–3.35 (m, 8H, 2 × NCH_2, 2 × $BocNHCH_2$), 4.67 (br s, 1H, NH), 5.43 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 22.42 ($CH(CH_3)_2$), 22.66 ($CH(CH_3)_2$), 25.00, 25.80, 26.21, 27.61, 28.38, 28.43, 29.99, 37.23, 39.94, 41.83, 41.88, 42.39, 45.38, 45.60, 47.46, 78.77 ($OC(CH_3)_3$), 79.25 ($OC(CH_3)_3$), 156.01 (NHCOOt-Bu), 156.15 (NHCOOtBu), 172.11 ($NCOCH_2$), 172.84 ($NCOCH_2$) ppm. ESI-MS *m/z* 430.2 $[M+H]^+$.

5.1.11. 2-Acetamido-*N*-[4-(*t*-butoxycarbonylamino)butyl]-*N*-[3-(*t*-butoxycarbonylamino) propyl]acetamide (14)

A mixture of **4** (0.69 g, 2 mmol), *N*-acetylglycine (0.234 g, 2 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 0.383 g, 2 mmol) and catalytic amount of 1-hydroxybenzotriazole (HOBt) in anhydrous dichloromethane (10 mL) was stirred at room temperature for 12 h. Then the reaction solution was quenched with water, the separated organic phase was washed with 5 % citric acid aqueous solution followed by 10 % ammonium chloride aqueous solution. Then the organic phase was dried over anhydrous $MgSO_4$ and concentrated under vacuum. The product was yielded as colourless oil, which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.37–1.72 (m, 24H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$), 1.98 (s, 3H, $NHCOCH_3$), 3.00–3.37 (m, 8H, 2 × NCH_2, 2 × $NHCH_2$), 3.96–3.99 (m, 2H, $COCH_2NH$), 4.64 (s, 1H, BocNH), 5.16 (s, 1H, BocNH), 6.57 (s, 1H, $NHCOCH_3$) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 23.01 ($NHCOCH_3$), 24.71, 25.73, 27.56, 28.37, 28.40, 28.42, 28.44, 41.18, 41.23, 42.94, 44.39, 45.62, 46.46, 79.13 ($OC(CH_3)_3$), 79.34 ($OC(CH_3)_3$), 156.04 (NHCOOtBu), 167.71 ($NHCOCH_2NH$), 168.20 ($NHCOCH_2NH$), 170.09 ($NHCOCH_3$) ppm. ESI-MS *m/z* 445.2 $[M+H]^+$.

5.1.12. 4-Methyl-*N*-[4-(*t*-butoxycarbonylamino)butyl]-*N*-[3-(*t*-butoxycarbonylamino)propyl] pentanamide (15)

This compound was prepared analogously to compound **14**. Compound **4** (0.5 g, 1.45 mmol), EDCI (0.28 g, 1.45 mmol) and 4-methylpentanoic acid (0.17 g, 1.45 mmol) were used as starting materials and reagents, respectively, and the crude product was used for the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.83 (d, 6H, $CH(CH_3)_2$), 1.36–1.59 (m, 27H, 2 × Boc, $NCH_2(CH_2)_2CH_2NH$, $NCH_2CH_2CH_2NH$, $CH_2CH(CH_3)_2$), 2.19–2.24 (m, 2H, $NCOCH_2$), 2.96–3.33 (m, 8H, 2 × NCH_2, 2 × $BocNHCH_2$), 4.84 (br s, 1H, NH), 5.46 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 22.32 ($CH(CH_3)_2$), 27.54, 27.82, 27.91, 28.33, 28.38, 31.03, 34.32, 34.44, 39.87, 42.44, 45.41, 45.61, 47.50, 78.72 ($OC(CH_3)_3$), 79.11 ($OC(CH_3)_3$), 156.07 (NHCOOtBu), 156.16 (NHCOOtBu), 173.19 ($NCOCH_2$), 173.80 ($NCOCH_2$) ppm. ESI-MS *m/z* 444.2 $[M+H]^+$.

Preparation and spectral data of quinazolinethiones 2,3-dihydropyrrolo[2,1-*b*]quinazoline-9(1*H*)-thione (**16a**) and 8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazoline-13(7*H*)-thione (**16b**) and their quinazolinone precursors have been described in detail recently.[19,21]

5.1.13. N^1-(2,3-Dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene)-N^3-(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl)-N^3-methylpropane-1,3-diamine (17)

To a solution of **5** (173 mg, 0.5 mmol) in anhydrous dichloromethane (5 mL) was added 4 M HCl in 1,4-dioxane (5 mL) and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated and the crude product hydrochloride was used in the next step without further purification. Alternatively, *N*-Me-*N*-(3-propylamino)propanediamine is also commercially available. A mixture of 2,3-dihydropyrrolo-[2,1-*b*]quinazoline-9(1*H*)-thione **16a**[19] (202 mg, 1 mmol), *N*-Me-*N*-(3-propylamino) propanediamine (0.08 mL, 0.5 mmol; alternatively 0.5 mmol of the hydrochloride obtained as described above), and sodium carbonate (318 mg, 3 mmol) was refluxed in ethanol (10 mL)/toluene (10 mL) in an 135 °C oil bath, and finely grinded mercury(II) bromide (0.72 g, 0.2 mmol) was added. The mixture was heated and stirred vigorously for 2 h. The mixture was then filtered and the filtrate concentrated. The crude product obtained was purified by column chromatography (chloroform/methanol/ammonia, 7:1:0.1). The pure compound was obtained as a yellow grease (36 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.83–1.88 (m, 4H, $CH_3N(CH_2CH_2CH_2N{=})_2$), 2.02–2.07 (m, 4H, 2 × NCH_2CH_2), 2.28 (s, 3H, NCH_3), 2.56 (t, 4H, J = 7.62 Hz, 2 × $N(CH_3)CH_2$), 2.93 (t, 4H, J = 7.86 Hz, 2 × CH_2C), 3.80 (t, 4H, J = 6.47 Hz, 2 × $CH_2N{=}$), 3.88 (t, 4H, J = 7.08 Hz, 2 × NCH_2), 7.06–7.12 (m, 2H, 2 × C(7)*H*), 7.39–7.41 (m, 4H, 2 × C(6, 8)*H*), 8.03 (d, 2H, J = 8.26 Hz, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 18.71, 30.77, 32.83, 42.82, 48.23, 48.61, 55.57, 119.83 (arom), 123.81 (arom), 126.78 (arom), 128.05 (arom), 131.58 (arom), 145.33 (arom), 148.85 (arom), 160.17 (arom) ppm. ESI-MS *m/z* 482 $[M+H]^+$. Elem. Anal. Calcd for ($C_{29}H_{35}N_7{\cdot}0.3CH_2Cl_2{\cdot}0.6CH_3OH$): C, 68.23; H, 7.28; N, 18.63. Found: C, 67.88; H, 7.45; N, 18.72.

5.1.14. *N*,*N*-Bis(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl) acetamide (18)

Compound **18** was synthesized analogously to compound **17** from compounds **7** (187 mg, 0.5 mmol) and **16a** (202 mg, 1 mmol). The crude product was purified by column chromatography (chloroform:methanol:ammonia, 7:1:0.1). The pure compound was obtained as a yellow grease (24 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.90–2.12 (m, 11H, $CH_3CON(CH_2CH_2CH_2N{=})_2$, 2 × NCH_2CH_2), 2.95 (t, 4H, J = 8.0 Hz, 2 × CH_2C), 3.44–3.54 (m, 4H, 2 × $N(COCH_3)CH_2$), 3.77–3.98 (m, 8H, 2 × $CH_2N{=}$, 2 × NCH_2), 7.12–7.20 (m, 2H, 2 × C(7)*H*), 7.44 (m, 4H, 2 × C(6, 8)*H*), 7.99–8.06 (m, 2H, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 19.17, 22.06, 32.65, 33.18, 44.30, 47.39, 47.80, 48.72, 120.01 (arom), 124.48 (arom), 127.42 (arom), 128.26 (arom), 132.37 (arom), 146.50 (arom), 149.25 (arom), 160.55 (arom), 170.94 ($COCH_3$) ppm. ESI-MS *m/z* 510.4 $[M+H]^+$. Elem. Anal. Calcd for ($C_{30}H_{35}N_7O{\cdot}H_2O{\cdot}$MeOH): C, 66.52; H, 7.38; N, 17.52. Found: C, 66.48; H, 7.71; N, 17.69.

5.1.15. N^1-Methyl-N^3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylidene)-N^1-(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)propane-1,3-diamine (19)

Compound **19** was synthesized analogously to compound **17** from 8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazoline-13(7*H*)-thione **16b**[19] (244 mg, 1 mmol) and *N*-Me-*N*-(3-propylamino)propanediamine (0.08 mL, 0.5 mmol; alternatively 0.5 mmol of its hydrochloride salt as described above). Purification by column chromatography (chloroform:methanol:ammonia, 9:1:0.1) yielded a colourless oil (16 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.33 (m, 4H, alkylene-*H*), 1.50 (m, 4H, alkylene-*H*), 1.76 (m, 4H, alkylene-*H*), 1.83–1.85 (m, 8H, alkylene-*H*), 2.28 (s, 3H, NCH_3), 2.56 (t, 4H, J = 6.05 Hz, 2 × $N(CH_3)CH_2$), 2.79 (t, 4H, J = 7.41 Hz, 2 × CH_2C), 3.79 (t, 4H, J = 5.94 Hz, 2 × $CH_2N{=}$), 4.17 (m, 4H, 2 × NCH_2), 7.05–7.08 (m, 2H, 2 × C(2)*H*), 7.35–7.40 (m, 4H, 2 × C(1, 3)*H*), 7.95 (d, 2H, J = 8.10 Hz, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 24.84, 27.02, 28.88, 31.03, 31.11, 36.50, 43.13, 44.38, 49.49, 56.14, 120.06 (arom), 123.90 (arom), 126.85 (arom), 128.30 (arom), 131.80 (arom), 145.96 (arom), 147.78 (arom),

160.68 (arom) ppm. ESI-MS *m/z* 566 $[M+H]^+$. Elem. Anal. Calcd for ($C_{35}H_{47}N_7 \cdot 0.8CHCl_3$): C, 65.02; H, 7.29; N, 14.83. Found: C, 64.77; H, 7.31; N, 14.55.

5.1.16. *N*,*N*-Bis(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)acetamide (20)

Compound **20** was synthesized analogously to compound **17** from compounds **7** (187 mg, 0.5 mmol) and **16a** (202 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 10:1:0.1) yielded a yellow grease (20 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.33–1.35 (m, 4H, alkylene-*H*), 1.50–1.51 (m, 4H, alkylene-*H*), 1.74–1.78 (m, 12H, alkylene-*H*), 2.06 (s, 3H, NCOC*H*$_3$), 2.80 (t, 4H, *J* = 6.24 Hz, 2 × C*H*$_2$C), 3.42 (t, 2H, *J* = 7.72 Hz, N(COCH$_3$)C*H*$_2$), 3.50 (t, 2H, *J* = 7.66 Hz, N(COCH$_3$)C*H*$_2$), 3.79 (t, 4H, *J* = 4.90 Hz, 2 × C*H*$_2$N═), 4.20 (m, 4H, 2 × NC*H*$_2$), 7.10–7.14 (m, 2H, 2 × C(2)*H*), 7.37–7.45 (m, 4H, 2 × C(1, 3)*H*), 7.96 (t, 2H, *J* = 7.65 Hz, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 22.06, 24.86, 24.89, 26.95, 27.04, 28.90, 31.04, 31.07, 31.93, 32.85, 36.59, 44.37, 44.47, 44.63, 47.74, 48.50, 48.16, 119.92 (arom), 120.03 (arom), 124.04 (arom), 124.12 (arom), 126.93 (arom), 127.16 (arom), 128.18 (arom), 128.32 (arom), 131.93 (arom), 132.12 (arom), 146.16 (arom), 146.79 (arom), 147.79 (arom), 147.85 (arom), 160.60 (arom), 160.69 (arom), 170.76 (*C*OCH$_3$) ppm. ESI-MS *m/z* 594.1 $[M+H]^+$. Elem. Anal. Calcd for ($C_{36}H_{47}N_7O \cdot 2HCl \cdot 5.3H_2O$): C, 56.73; H, 7.88; N, 12.86. Found: C, 57.03; H, 7.50; N, 12.48.

5.1.17. N^1-(2,3-Dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylidene)-N^4-(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl)-N^4-methylbutane-1,4-diamine (21)

Compound **21** was synthesized analogously to compound **17** from compounds **6** (180 mg, 0.5 mmol) and **16b** (202 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 9:1:0.1) yielded a yellow grease (20 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.67–1.75 (m, 4H, alkylene-*H*), 1.90–1.92 (m, 2H, alkylene-*H*), 2.02–2.06 (m, 4H, 2 × NCH$_2$C*H*$_2$), 2.39 (s, 3H, NC*H*$_3$), 2.60–2.72 (m, 4H, 2 × N(CH$_3$)C*H*$_2$), 2.93 (t, 4H, *J* = 7.99 Hz, 2 × C*H*$_2$C), 3.77–3.83 (m, 4H, *J* = 6.24 Hz,2 × C*H*$_2$N═), 3.86–3.90 (m, 4H, *J* = 7.03 Hz, 2 × NC*H*$_2$), 7.12–7.16 (m, 2H, 2 × C(7)*H*), 7.40–7.45 (m, 4H, 2 × C(6, 8)*H*), 8.01 (d, 2H, *J* = 8.19 Hz, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 17.71, 29.87, 31.80, 40.77, 47.32, 47.37, 49.15, 54.01, 56.05, 118.65 (arom), 118.69 (arom), 122.91 (arom), 122.99 (arom), 125.86 (arom), 127.01 (arom), 130.74 (arom), 130.80 (arom), 144.60 (arom), 144.82 (arom), 147.82 (arom), 147.87 (arom), 159.16 (arom), 159.25 (arom) ppm. ESI-MS *m/z* 496.4 $[M+H]^+$. Elem. Anal. Calcd for ($C_{30}H_{37}N_7 \cdot 1.5CHCl_3 \cdot 2.5MeOH$): C, 58.08; H, 7.17; N, 13.94. Found: C, 57.73; H, 6.85; N, 13.93.

5.1.18. *N*-(4-(2,3-Dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylid - eneamino)butyl)-*N*-(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl)acetamide (22)

Compound **22** was synthesized analogously to compound **17** from compounds **8** (194 mg, 0.5 mmol) and **16a** (202 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 9:1:0.1) yielded a yellow grease (31 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.64–1.76 (m, 4H, alkylene-*H*), 1.88–1.95 (m, 2H, alkylene-*H*), 2.03–2.08 (m, 7H, 2 × NCH$_2$C*H*$_2$, NCOC*H*$_3$), 2.92–2.96 (m, 4H, 2 × C*H*$_2$C), 3.28–3.50 (m, 4H, 2 × N(COCH$_3$)C*H*$_2$), 3.77–3.81 (m, 4H, 2 × C*H*$_2$N═), 3.88–3.98 (m, 4H, 2 × NC*H*$_2$), 7.14–7.18 (m, 2H, 2 × C(7)*H*), 7.44–7.47 (m, 4H, 2 × C(6, 8)*H*), 7.80–8.02 (m, 2H, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 17.76, 20.66, 24.58, 25.82, 29.37, 31.73, 31.78, 42.90, 44.58, 44.90, 45.66, 46.36, 47.30, 48.17, 49.99, 119.67 (arom), 119.73 (arom), 123.85 (arom), 123.94 (arom), 126.87 (arom), 126.97 (arom), 128.09 (arom), 128.20 (arom), 131.75 (arom), 131.88 (arom), 145.63 (arom), 148.88 (arom), 148.91 (arom), 160.13 (arom), 160.19 (arom), 170.16 (*C*OCH$_3$), 170.29 (*C*OCH$_3$) ppm. ESI-MS *m/z* 524.7 $[M+H]^+$. Elem. Anal. Calcd for ($C_{31}H_{37}N_7O \cdot MeOH$): C, 69.16; H, 7.44; N, 17.64. Found: C, 69.28; H, 7.14; N, 17.69.

5.1.19. *N*-(4-(2,3-Dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)butyl)-*N*-(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl) propionamide (25)

Compound **25** was synthesized analogously to compound **17** from compounds **9** (201 mg, 0.5 mmol) and **16a** (202 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 16:1:0.1) yielded a yellow grease (37 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.07 (t, 3H, *J* = 7.42 Hz, NCOCH$_2$C*H*$_3$), 1.61–1.74 (m, 4H, alkylene-*H*), 1.87–1.88 (m, 2H, alkylene-*H*), 1.96–2.09 (m, 4H, 2 × NCH$_2$C*H*$_2$), 2.24–2.38 (dq, J_1 = 7.43 Hz, J_2 = 30.40 Hz, NCOC*H*$_2$), 2.87–2.95 (m, 4H, 2 × C*H*$_2$C), 3.25–3.50 (m, 4H, 2 × N(COCH$_2$CH$_3$)C*H*$_2$), 3.74–3.77 (m, 4H, 2 × C*H*$_2$N═), 3.84–3.89 (m, 4H, 2 × NC*H*$_2$), 7.10–7.16 (m, 2H, 2 × C(7)*H*), 7.37–7.44 (m, 4H, 2 × C(6, 8)*H*), 7.98–8.01 (m, 2H, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 9.72 (NCOCH$_2$*C*H$_3$), 9.80 (NCOCH$_2$CH$_3$), 18.66, 25.70, 26.36, 26.38, 26.91, 30.49, 30.74, 31.29, 32.39, 32.77, 44.13, 45.69, 45.81, 47.43, 48.10, 48.21, 50.05, 50.35, 119.64 (arom), 119.77 (arom), 123.84 (arom), 123.97 (arom), 126.77 (arom), 126.98 (arom), 127.83 (arom), 128.02 (arom), 131.60 (arom), 131.85 (arom), 145.95 (arom), 148.85 (arom), 160.08 (arom), 160.20 (arom), 173.53 (*C*OCH$_3$), 173.38 (*C*OCH$_3$) ppm. ESI-MS *m/z* 538.4 $[M+H]^+$. Elem. Anal. Calcd for ($C_{36}H_{49}N_7 \cdot H_2O$): C, 72.32; H, 8.60; N, 16.40. Found: C, 72.38; H, 8.86; N, 16.57.

5.1.20. *N*-(4-(2,3-Dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)butyl)-*N*-(3-(2,3-dihydropyrrolo[2,1-*b*]quinazolin-9(1*H*)-ylideneamino)propyl) methacrylamide (27)

Compound **27** was synthesized analogously to compound **17** from compounds **10** (207 mg, 0.5 mmol) and **16a** (202 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 20:1:0.1) yielded a yellow grease (29 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.59–2.09 (m, 13H, alkylene-*H*, 2 × NCH$_2$C*H*$_2$, CH$_2$═CC*H*$_3$), 2.91 (t, *J* = 5.80 Hz, 2 × C*H*$_2$C), 3.35–3.52 (m, 4H, 2 × C*H*$_2$N(COC(CH$_3$)═CH$_2$)), 3.76–3.79 (m, 4H, 2 × C*H*$_2$N═), 3.86–3.88 (m, 4H, 2 × NC*H*$_2$), 4.92–5.02 (dd, 2H, J_1 = 10.64 Hz, J_2 = 29.50 Hz, COC═C*H*$_2$), 7.12–7.15 (m, 2H, 2 × C(7)*H*), 7.41–7.43 (m, 4H, 2 × C(6, 8)*H*), 7.98–8.02 (m, 2H, 2 × C(5)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 14.02 (COC(*C*H$_3$)═CH$_2$), 18.70, 20.78, 22.96, 23.75, 25.29, 26.83, 28.91, 30.36, 30.47, 30.89, 32.41, 32.79, 38.73, 42.32, 44.19, 46.67, 47.68, 48.25, 48.71, 50.22, 114.17 (COC═*C*H$_2$), 119.67 (arom), 123.86 (arom), 123.96 (arom), 125.27 (arom), 126.89 (arom), 127.93 (arom), 129.00 (arom), 131.71 (arom), 131.81 (arom), 141.60 (*C*OC═CH$_2$), 148.88 (arom), 148.91 (arom), 160.11 (arom), 160.19 (arom), 172.60 (*C*OC═CH$_2$) ppm. ESI-MS *m/z* 550.5 $[M+H]^+$. Elem. Anal. Calcd for ($C_{38}H_{51}N_7O \cdot 0.9CHCl_3$): C, 64.06; H, 7.17; N, 13.44. Found: C, 64.05; H, 6.87; N, 13.48.

5.1.21. (*Z*)-N^1-Methyl-N^4-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylidene)-N^1-(3-((*E*)-8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-butane-1,4-diamine (23)

Compound **23** was synthesized analogously to compound **17** from compounds **6** (180 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (dichloromethane/methanol/triethylamine, 30:1:0.05) yielded a yellow grease (22 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.31–1.32 (m, 4H, alkylene-*H*), 1.49 (m, 4H, alkylene-*H*), 1.64–1.84 (m, 14H, alkylene-*H*), 2.28 (s, 3H, NC*H*$_3$), 2.48–2.60 (m, 4H, 2 × N(CH$_3$)C*H*$_2$), 2.76–2.80 (m, 4H, 2 × C*H*$_2$C), 3.77–3.81 (m, 4H, 2 × C*H*$_2$N═), 4.13–4.19 (m, 4H, 2 × NC*H*$_2$), 7.07–7.11 (m, 2H, 2 × C(2)*H*), 7.35–7.42 (m, 4H, 2 × C(1, 3)*H*), 7.97 (t, 2H, *J* = 7.63 Hz, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 24.41, 24.43, 26.60, 26.62, 28.49, 28.53, 30.57,

30.65, 31.40, 36.16, 42.76, 43.93, 44.04, 49.22, 50.92, 55.88, 58.00, 119.67 (arom), 119.71 (arom), 123.44 (arom), 123.51 (arom), 126.51 (arom), 126.54 (arom), 127.91 (arom), 131.35 (arom), 131.43 (arom), 145.43 (arom), 145.88 (arom), 147.48 (arom), 147.50 (arom), 160.128 (arom), 160.32 (arom) ppm. ESI-MS *m/z* 580.5 $[M+H]^+$. Elem. Anal. Calcd for ($C_{32}H_{39}N_7O\cdot1.3CH_2Cl_2$): C, 61.71; H, 6.47; N, 15.13. Found: C, 61.99; H, 6.76; N, 14.89.

5.1.22. *N*-(4-(8,9,10,11-Tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,11-tetrahydro--6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-acetamide (24)

Compound **24** was synthesized analogously to compound **17** from compounds **8** (194 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 25:1:0.1) yielded a yellow grease (20 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.33–1.37 (m, 4H, alkylene-*H*), 1.51 (m, 4H, alkylene-*H*), 1.64–1.77 (m, 14H, alkylene-*H*), 2.06 (s, 3H, $NCOCH_3$), 2.80–2.82 (m, 4H, 2 × CH_2C), 3.25–3.49 (m, 4H, $N(COCH_3)CH_2$), 3.79–3.81 (m, 4H, 2 × CH_2N=), 4.17–4.21 (m, 4H, 2 × NCH_2), 7.12–7.13 (m, 2H, 2 × C(2)*H*), 7.41–7.42 (m, 4H, 2 × C(1, 3)*H*), 7.95–7.97 (m, 2H, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 22.01, 24.84, 26.15, 26.94, 27.00, 27.39, 28.89, 31.04, 32.84, 36.49, 44.41, 44.71, 46.06, 47.37, 48.51, 48.99, 49.72, 51.00, 51.21, 119.90 (arom), 124.00 (arom), 124.14 (arom), 127.00 (arom), 127.15 (arom), 128.17 (arom), 128.29 (arom), 131.98 (arom), 132.11 (arom), 142.79 (arom), 147.65 (arom), 147.80 (arom), 160.57 (arom), 160.80 (arom), 170.62 ($COCH_3$), 170.67 ($COCH_3$) ppm. ESI-MS *m/z* 607.6 $[M+H]^+$. Elem. Anal. Calcd for ($C_{39}H_{52}N_8O_2\cdot1.4CH_2Cl_2$): C, 61.79; H, 6.30; N, 14.66. Found: C, 61.90; H, 6.25; N, 14.63.

5.1.23. *N*-(4-(8,9,10,11-Tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-propionamide (26)

Compound **26** was synthesized analogously to compound **17** from compounds **9** (201 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 25:1:0.1) yielded a yellow grease (26 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.05–1.09 (dt, 3H, J_1 = 7.43 Hz, J_2 = 2.74 Hz, $NCOCH_2CH_3$), 1.32–1.35 (m, 4H, alkylene-*H*), 1.45–1.52 (m, 4H, alkylene-*H*), 1.62–1.85 (m, 14H, alkylene-*H*), 2.24–2.34 (dq, 2H, J_1 = 19.23 Hz, J_2 = 7.44 Hz, $NCOCH_2CH_3$), 2.76–2.82 (m, 4H, 2 × CH_2C), 3.23–3.48 (m, 4H, 2 × $N(COCH_2CH_3)CH_2$), 3.75–3.79 (m, 4H, 2 × CH_2N=), 4.14–4.18 (m, 4H, 2 × NCH_2), 7.07–7.13 (m, 2H, 2 × C(2)*H*), 7.35–7.43 (m, 4H, 2 × C(1, 3)*H*), 7.92–7.97 (m, 2H, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 9.79 ($NCOCH_2CH_3$), 24.43, 24.46, 25.82, 26.36, 26.52, 26.57, 26.62, 27.08, 28.47, 28.50, 30.62, 30.78, 31.00, 31.51, 32.55, 36.13, 43.89, 43.96, 43.99, 44.48, 45.79, 45.79, 45.98, 48.13, 48.29, 48.67, 50.58, 50.87, 119.51 (arom), 119.57 (arom), 123.47 (arom), 123.50 (arom), 123.57 (arom), 123.67 (arom), 126.48 (arom), 126.55 (arom), 126.64 (arom), 126.76 (arom), 127.75 (arom), 127.77 (arom), 127.91 (arom), 131.37 (arom), 131.46 (arom), 131.51 (arom), 131.65 (arom), 145.74 (arom), 145.87 (arom), 147.47 (arom), 160.13 (arom), 160.21 (arom), 160.26 (arom), 160.30 (arom), 173.37 ($COCH_2CH_3$), 173.48 ($COCH_2CH_3$) ppm. ESI-MS *m/z* 622.5 $[M+H]^+$. Elem. Anal. Calcd for ($C_{37}H_{49}N_7O\cdot0.9CH_2Cl_2$): C, 66.52; H, 7.48; N, 14.33. Found: C, 66.13; H, 7.53; N, 14.23.

5.1.24. *N*-(4-(8,9,10,11-Tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-methacrylamide (28)

Compound **28** was synthesized analogously to compound **17** from compounds **10** (207 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification of the crude product by column chromatography (chloroform:methanol:ammonia, 30:1:0.1) yielded a yellow grease (27 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.31–1.88 (m, 25H, 2 × $NCH_2(CH_2)_4$, =NCH_2CH_2, =$NCH_2CH_2CH_2$, $NCOC(CH_3)$=CH_2), 2.76–2.79 (m, 4H, 2 × CH_2C), 3.32–3.51 (m, 4H, 2 × $CH_2N(COC(CH_3)$=$CH_2)$), 3.76–3.77 (m, 4H, 2 × CH_2N=), 4.13–4.20 (m, 4H, 2 × NCH_2), 4.93–5.01 (dd, 2H, J_1 = 5.08 Hz, J_2 = 27.39 Hz, COC=CH_2), 7.07–7.12 (m, 2H, 2 × C(2)*H*), 7.36–7.42 (m, 4H, 2 × C(1, 3)*H*), 7.92–7.96 (m, 2H, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 14.67 ($COC(CH_3)$=CH_2), 21.43, 20.78, 24.40, 24.43, 26.55, 26.58, 28.47, 30.61, 30.78, 36.11, 43.91, 48.39, 48.58, 50.71, 114.17 (COC=CH_2), 119.55 (arom), 119.61 (arom), 123.47 (arom), 123.60 (arom), 126.13 (arom), 126.56 (arom), 127.81 (arom), 128.18 (arom), 128.77 (arom), 128.99 (arom), 131.42 (arom), 131.53 (arom), 141.56 (COC=CH_2), 147.45 (arom), 147.47 (arom), 160.15 (arom), 160.24 (arom), 172.58 (COC=CH_2) ppm. ESI-MS *m/z* 634.6 $[M+H]^+$. Elem. Anal. Calcd for ($C_{39}H_{51}N_7O\cdot1.4CH_2Cl_2$): C, 64.46; H, 7.20; N, 13.02. Found: C, 64.21; H, 7.36; N, 12.98.

5.1.25. 2-Acetamido-*N*-(4-(8,9,10,11-tetrahydro-6*H*-azocino-[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,-11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)acetamide (29)

Compound **29** was synthesized analogously to compound **17** from compounds **14** (223 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification of the crude product by column chromatography (chloroform:methanol:ammonia, 18:1:0.1) yielded a yellow grease (18 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.32–1.86 (m, 22H, 2 × $NCH_2(CH_2)_4$, =NCH_2CH_2, =$NCH_2CH_2CH_2$), 1.98 (s, 3H, $NCOCH_3$), 2.77–2.83 (m, 4H, 2 × CH_2C), 3.22–3.52 (m, 4H, 2 × CH_2NCOCH_2), 3.77–3.80 (m, 4H, 2 × $CH2N$=), 4.00–4.22 (m, 6H, $NCOCH_2NH$, 2 × NCH_2), 6.60–6.62 (m, 1H, $NHCOCH_3$), 7.10–7.15 (m, 2H, 2 × C(2)*H*), 7.41–7.44 (m, 4H, 2 × C(1, 3)*H*), 7.93–7.96 (m, 2H, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 14.20 ($NCOCH_3$), 23.08, 24.40, 26.54, 26.57, 28.46, 30.62, 30.64, 36.02, 41.46, 44.00, 44.12, 44.92, 46.01, 47.30, 47.91, 48.45, 50.60, 50.70, 119.41 (arom), 123.67 (arom), 123.72 (arom), 126.42 (arom), 126.52 (arom), 126.70 (arom), 127.74 (arom), 127.79 (arom), 127.83 (arom), 131.61 (arom), 131.72 (arom), 146.59 (arom), 147.25 (arom), 147.37 (arom), 160.31 (arom), 167.50 ($NCOCH_2NH$), 167.66 ($NCOCH_2NH$), 169.95 ($NCOCH_3$), 170.01 ($NCOCH_3$) ppm. ESI-MS *m/z* 665.7 $[M+H]^+$. Elem. Anal. Calcd for ($C_{39}H_{52}N_8O_2\cdot1.4CH_2Cl_2$): C, 61.91; H, 7.05; N, 14.30. Found: C, 62.31; H, 7.12; N, 13.93.

5.1.26. *N*-(4-(8,9,10,11-Tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-pivalamide (30)

Compound **30** was synthesized analogously to compound **17** from compounds **11** (215 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 32:1:0.1) yielded a yellow grease (19 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.22 (s, 9H, $COC(CH_3)_3$), 1.32 (m, 4H, alkylene-*H*), 1.49–1.91 (m, 18H, alkylene-*H*), 2.76–2.79 (m, 4H, 2 × CH_2C), 3.35–3.49 (m, 4H, 2 × $CH_2NCOtBu$), 3.77–3.79 (m, 4H, 2 × CH_2N=), 4.12–4.25 (m, 4H, 2 × NCH_2), 7.07 (m, 2H, 2 × C(2)*H*), 7.36–7.41 (m, 4H, 2 × C(1, 3)*H*), 7.93–7.98 (m, 2H, 2 × C(4)*H*) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 24.42, 26.53, 26.60, 28.46, 28.49, 28.64, 30.62, 31.01, 36.14, 36.19, 39.02, 43.78, 43.91, 48.90, 50.82, 119.63 (arom), 123.46 (arom), 123.57 (arom), 126.58 (arom), 126.69 (arom), l28.78 (arom), 131.41 (arom), 131.51 (arom), 145.61 (arom), 145.97 (arom), 147.50 (arom), 160.17 (arom), 160.26 (arom), 177.02 ($NCOtBu$) ppm. ESI-MS *m/z* 650.7 $[M+H]^+$. Elem. Anal. Calcd for ($C_{40}H_{55}N_7O\cdot MeOH$): C, 72.21; H, 8.72; N, 14.38. Found: C, 72.19; H, 8.80; N, 14.27.

5.1.27. *N*-(4-(8,9,10,11-Tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)-pentanamide (31)

Compound **31** was synthesized analogously to compound **17** from compounds **12** (215 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 32:1:0.1) yielded a yellow grease (35 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.84 (t, 3H, J = 7.32 Hz, NCO$(CH_2)_3CH_3$), 1.18–1.85 (m, 26H, 2 × NCH$_2$(CH_2)$_4$, =NCH$_2$CH_2, =NCH$_2$CH_2CH_2, NCOCH$_2$(CH_2)$_2$-CH_3), 2.22–2.30 (m, 2H, NCOCH_2), 2.78–2.81 (m, 4H, 2 × CH_2C), 3.24–3.48 (m, 4H, 2 × CH_2NCO), 3.77–3.80 (m, 4H, 2 × CH_2N=), 4.13–4.24 (m, 4H, 2 × NCH_2), 7.09–7.14 (m, 2H, 2 × C(2)H), 7.39–7.42 (m, 4H, 2 × C(1,3)H), 7.93–7.96 (m, 2H, 2 × C(4)H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 13.92 (NCO$(CH_2)_3CH_3$), 13.97, 22.63, 24.42, 25.79, 26.52, 26.56, 26.60, 27.70, 27.72, 28.46, 28.52, 30.59, 30.77, 30.96, 32.59, 32.87, 32.99, 36.07, 43.92, 43.96, 44.01, 44.36, 45.71, 46.07, 48.12, 48.37, 48.64, 50.58, 50.82, 119.49 (arom), 119.55 (arom), 119.62 (arom), 123.49 (arom), 123.58 (arom), 126.43 (arom), 126.50 (arom), 126.61 (arom), 126.73 (arom), 127.72 (arom), 127.87 (arom), 131.36 (arom), 131.43 (arom), 131.47 (arom), 131.61 (arom), 146.35 (arom), 147.37 (arom), 147.44 (arom), 147.46 (arom), 160.08 (arom), 160.16 (arom), 160.22 (arom), 160.26 (arom), 172.77 (NCO$(CH_2)_3CH_3$), 172.80 (NCO$(CH_2)_3CH_3$) ppm. ESI-MS m/z 650.7 [M+H]$^+$. Elem. Anal. Calcd for ($C_{40}H_{55}N_7O{\cdot}CH_2Cl_2$): C, 67.01; H, 7.82; N, 13.34. Found: C, 66.80; H, 7.60; N, 13.28.

5.1.28. 3-Methyl-*N*-(4-(8,9,10,11-tetrahydro-6*H*-azocino-[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,-10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)butanamide (32)

Compound **32** was synthesized analogously to compound **17** from compounds **13** (215 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 30:1:0.1 as eluent) yielded a yellow grease (28 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.87 (d, 6H, 2 × CH_3), 1.32–1.88 (m, 23H, 2 × NCH$_2$(CH_2)$_4$, =NCH$_2$CH_2, =NCH$_2$CH_2CH_2, CH(CH$_3$)$_2$), 2.12–2.15 (m, 2H, NCOCH_2), 2.75–2.81 (m, 4H, 2 × CH_2C), 3.24–3.48 (m, 4H, 2 × CH_2NCO), 3.75–3.78 (m, 4H, 2 × CH_2N=), 4.12–4.24 (m, 4H, 2 × NCH_2), 7.06–7.13 (m, 2H, 2 × C(2)H), 7.35–7.42 (m, 4H, 2 × C(1, 3)H), 7.92–7.95 (m, 2H, 2 × C(4)H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 22.72 (CHCH3), 22.73 (CHCH3), 24.40, 25.82, 25.84, 25.87, 26.54, 26.57, 26.60, 28.45, 30.61, 30.80, 31.00, 32.63, 36.11, 41.89, 42.08, 43.95, 45.74, 46.14, 48.12, 48.44, 48.66, 50.62, 50.84, 119.50 (arom), 119.57 (arom), 123.50 (arom), 123.58 (arom), 123.66 (arom), 126.46 (arom), 126.52 (arom), 126.63 (arom), 126.74 (arom), 127.76 (arom), 127.90 (arom), 131.36 (arom), 131.44 (arom), 131.49 (arom), 131.64 (arom), 145.70 (arom), 145.80 (arom), 147.42 (arom), 147.45 (arom), 147.49 (arom), 160.10 (arom), 160.17 (arom), 160.24 (arom), 160.28 (arom), 172.11 (NCOCH$_2$), 172.16 (NCOCH$_2$) ppm. ESI-MS m/z 650.7 [M+H]$^+$. Elem. Anal. Calcd for ($C_{40}H_{55}N_7O{\cdot}1.2CH_2Cl_2$): C, 65.82; H, 7.70; N, 13.04. Found: C, 65.67; H, 7.48; N, 13.13.

5.1.29. 4-Methyl-*N*-(4-(8,9,10,11-tetrahydro-6*H*-azocino-[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)butyl)-*N*-(3-(8,9,-10,11-tetrahydro-6*H*-azocino[2,1-*b*]quinazolin-13(7*H*)-ylideneamino)propyl)pentanamide (33)

Compound **33** was synthesized analogously to compound **17** from compounds **15** (222 mg, 0.5 mmol) and **16b** (244 mg, 1 mmol). Purification by column chromatography (chloroform:methanol:ammonia, 32:1:0.05) yielded a yellow grease (18 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.82–0.83 (d, 6H, CH(CH_3)$_2$), 1.32–1.86 (m, 25H, 2 × NCH$_2$(CH_2)$_4$, =NCH$_2$CH_2, =NCH$_2$CH_2CH_2, CH_2CH(CH$_3$)$_2$), 2.22–2.30 (m, 2H, NCOCH_2), 2.77–2.82 (m, 4H, 2 × CH_2C), 3.24–3.48 (m, 4H, 2 × CH_2NCO), 3.75–3.80 (m, 4H, 2 × CH_2N=), 4.13–4.28 (m, 4H, 2 × NCH_2), 7.08–7.14 (m, 2H, 2 × C(2)H), 7.36–7.44 (m, 4H, 2 × C(1, 3)H), 7.93–7.97 (m, 2H, 2 × C(4)H) ppm. ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 22.42 (CHCH$_3$), 22.48 (CHCH$_3$), 24.42, 26.54, 26.58, 27.96, 28.00, 28.48, 30.61, 32.61, 34.48, 34.51, 36.01, 43.95, 44.03, 45.76, 46.11, 48.19, 48.41, 48.65, 50.62, 50.87, 119.51 (arom), 123.52 (arom), 123.61 (arom), 123.69 (arom), 126.51 (arom), 126.62 (arom), 126.73 (arom), 127.75 (arom), 127.91 (arom), 131.52 (arom), 131.67 (arom), 147.43 (arom), 160.15 (arom), 160.29 (arom), 173.03 (NCOCH$_2$) ppm. ESI-MS m/z 664.7 [M+H]$^+$. Elem. Anal. Calcd for ($C_{41}H_{57}N_7O{\cdot}1.1MeOH{\cdot}0.2CH_2Cl_2$): C, 70.94; H, 8.70; N, 13.69. Found: C, 70.56; H, 8.42; N, 13.41.

5.2. Cholinesterase inhibition

AChE (E.C.3.1.1.7, Type VI-S, from Electric Eel) and BChE (E.C.3.1.1.8, from equine serum) were purchased from Sigma-Aldrich, UK. 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB), acetylthiocholine (ATC) and butyrylthiocholine (BTC) were also purchased from Sigma–Aldrich, UK. UV measurements were performed on Varian Cary 50 Scan UV–vis spectrometer and 4 mL disposable cuvettes were used for the testing. As references for ChE inhibition the established drugs galanthamine and tacrine were used. Galanthamine hydrobromide was purchased from JANSSEN-CILAG GmbH, Neuss, Germany. Tacrine hydrochloride was purchased from Sigma–Aldrich, UK.

The assay was performed as described in the following procedure:[19,21] A 3.32×10^{-3} M stock solution of the test compound was prepared in 50% ethanol/buffer (pH 8), and 100 μL of this solution were applied in 3.32 mL of final volume in the testing cuvette, so that the highest concentration of the test compounds applied in the assay was 10^{-4} M (final concentration). In order to obtain an inhibition curve, at least five different concentrations (normally in the range of 10^{-4}–10^{-9} M) of the test compound were measured at 25 °C at 412 nm, each concentration in triplicate.[19,21] For buffer preparation, 1.36 g of potassium dihydrogen phosphate (10 mmol) were dissolved in 100 mL of water and adjusted with NaOH to pH 8.0 ± 0.1. AChE solution was prepared to give 2.5 units/mL in 1.4 mL aliquots whereas for BChE the concentration was 25 units/mL in 1.4 mL aliquots because of BChE's lower activity. Furthermore, 0.01 M DTNB solution, 0.075 M ATC and BTC solutions, respectively, were used. A cuvette containing 3.0 mL of phosphate buffer, 100 μL of the respective enzyme (AChE/BChE) and 100 μL of the test compound solution was allowed to stand for 3.0 min accurately, then 100 μL of DTNB were added, then after another 1.5 min the reaction was started by addition of 20 μL of the substrate solution (ATC or BTC according to corresponding enzyme). The solution was mixed immediately, and exactly 2.5 min after the addition of substrate the absorption was measured using UV–vis spectrometer. For the reference value 100 μL of water replaced the test compound solution. For determining the blank value, additionally 100 μL of water replaced the enzyme solution.

The inhibition curve was obtained by plotting percentage enzyme activity (100 % for the reference) versus logarithm of test compound concentration using GraphPad Prism software (version 5.01).

5.3. Molecular modelling

Homology modelling of the enzymes based on the crystal structure of Torpedo californica AChE (2CEK) was conducted using Molecular Operating Environment (MOE) software[33] with a default protocol. The ligands were docked using the Induced Fit docking protocol of the Schrödinger software[34] with the Extra Precision

option. The ligands in advance were prepared for the docking procedure using the LigPrep module[35] of the Schrödinger software. The best poses were selected based on the energy score and the general position of bivalent ligands in the available crystal structures (PDB: code 2CMF, 2CKM and 2CEK). The hydrophobic, acceptor and donor regions of the binding site located at the radius of 4 Å around the ligand were calculated using the Site Map.[36] The OPLS2005 force fields were used in all calculations. The pictures were generated using MOE and Schrödinger software.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.034. These data include MOL files and InChiKeys of the most important compounds described in this article.

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