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PAPER

Clicked tacrine conjugates as acetylcholinesterase and β-amyloid directed compounds†

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The multifaceted nature of Alzheimer's disease (AD) has led to the development of multi-targeted compounds based on the classical AD drug, tacrine, first known to inhibit the acetylcholine-degrading enzyme acetylcholinesterase (AChE). In the present work, we explore the potentiality of multimers of tacrine in this field. The synthesis using the so-called "click chemistry" and the *in vitro* study of the conjugates are described. Two or four copies of the tacrine molecule are "clicked" on a constrained cyclopeptide template proven to be a convenient tool for multimeric presentation. The multimers significantly inhibit self-induced amyloid fibril formation from $A\beta_{40}$ at low inhibitor to Aβ molar ratios at which the tacrine monomer is fully inactive (Thioflavin T assays and AFM observation). Moreover, they have the capacity to bind to $A\beta_{40}$ fibrils (SPR assays) while retaining the AChE inhibitory activity of the parent tacrine.

Introduction

Alzheimer's disease (AD), the most common form of dementia, is a progressive neurodegenerative disease characterized by a loss of memory and cognition. The neuropathological hallmarks of AD are extracellular deposits of β-amyloid peptide (Aβ), intracellular neurofibrillary tangles (NTF), and loss of cholinergic neurons.[1] The progressive accumulation of Aβ aggregates is widely believed to be fundamental to the initial development of the disease and to trigger a cascade of events such as neurotoxicity, oxidative damage, and inflammation leading to neuronal dysfunction and death.[2,3] Currently no treatment is available to cure AD and clinical treatments have only palliative effects, such as acetylcholinesterase inhibitors AChEIs (tacrine, donepezil, rivastigmine, galatamine) which all restore the cholinergic deficit, or *N*-methyl-D-aspartate (NMDA) receptor antagonists (memantine), which act as neuroprotective agents by blocking excessive glutamatergic stimulation.[4] Due to the multi-pathogenesis of AD, one of the current strategies is to develop multi-potent drugs that can target Aβ deposits and other biological dysfunctions implicated in the disease. In this context, tacrine derivatives have been explored as multi-target-directed ligands (MTDLs).[5,6] Indeed, tacrine is known to stabilize acetylcholine levels in the synaptic cleft by inhibiting the neurotransmitter-degrading enzyme AChE. Even when used to improve the cognition in AD patients, tacrine is not without side effects. However due to its potential as an AChEI, drugs derived from tacrine having additional anti AD activities are of interest.[7–15] Several of them are "dual binding site" AChEIs. Able to simultaneously interact with both the catalytic and the peripheral anionic site of the enzyme, they have the additional capability of inhibiting the AChE-induced amyloid aggregation through AChE peripheral side blockage.[16] Lipocrine,[13] a heterodimer of lipoic acid and tacrine, is one of the first examples reported in the literature, the lipoyl part acting both as a ligand for the peripheral site and as an antioxidant. A large number of tacrine hybrids have been designed since. Recently a series of donepezil-tacrine molecules that inhibit cholinesterases in the subnanomolar range and block the AChE-induced Aβ aggregation have been reported.[8] Tacrine homodimers have also been reported as promising MTDLs. Indeed, bis(7)-tacrines, having a superior AChE inhibitory activity than tacrine alone, have shown multiple activities against AD: reduction of the Aβ production by inhibiting the BACE-1 β-secretase, and Aβ-induced neuronal apoptosis[10,12] as well as inhibition of AChE-induced amyloid aggregation.[7] Nevertheless, although tacrine, as a lipophilic heterocyclic molecule, displays a suitable profile to interact with Aβ oligomers or fibrils, only a few tacrine derivatives have been investigated as inhibitors of self-induced Aβ aggregation or as markers of Aβ fibrils.[9,14,17] We propose in this study to contribute to this field by designing conjugates bearing two or four copies of the tacrine molecule (Fig. 1) to evaluate their activity as interaction partners for the β-amyloid aggregation-prone species or for Aβ oligomers or fibrils.

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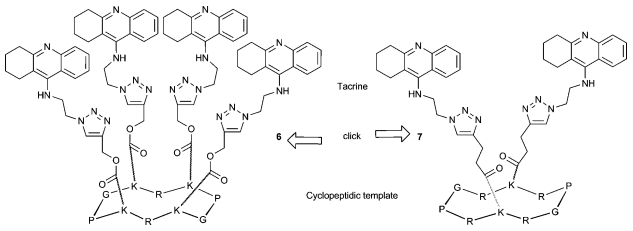


Fig. 1 Tacrine conjugates.

In previous works, we have shown the efficiency of combining potential Aβ-recognition domains on a cyclopeptidic template for preventing amyloid fibril formation.[18,19] To create an efficient binding domain, the strategy is based on the multimeric presentation of aromatic/hydrophobic compounds, enabling them to interact within the grooves created by β-sheets and also with hydrophobic and aromatic parts of the misfolded Aβ.[20] This binding domain is presented at the surface of an arginine-rich cyclopeptide used as a disturbing element. In this way, we have designed a tetramer of quinacrine as well as dimers of $A\beta_{16-21}$ fragment and curcumin, and we have shown their ability to interfere with $A\beta_{40}$ fibril formation.[18,19]

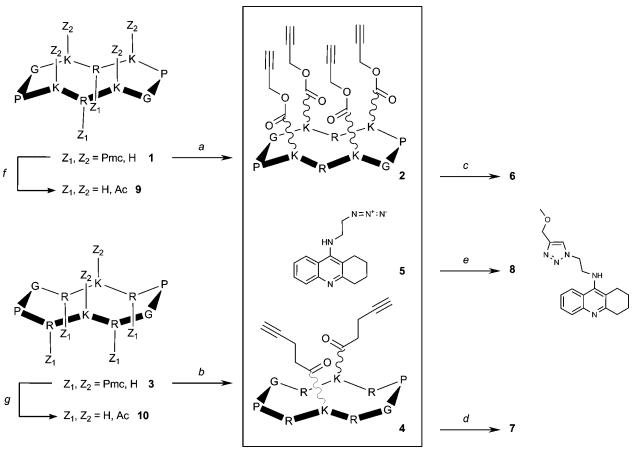
These previous results and the potentiality of tacrine molecule in AD events encouraged us to apply our strategy to prepare multimers of tacrine. The designed tacrine conjugates differ in the number of tacrine moieties and the nature of their scaffold (Fig. 1, compounds **6** and **7**). In the present work, we describe the synthesis of the conjugates using "click chemistry", their Aβ fibril binding ability and their evaluation as inhibitors of self-induced $A\beta_{40}$ fibril formation. The capacity of the conjugates to retain the AChE inhibitory effect of the parent tacrine was also verified using enzymatic assays.

Results

Synthesis

The synthesis of conjugates **6** and **7** is summarized in Scheme 1. The tacrine subunits were attached as azide derivatives **5** on the alkynyl cyclic scaffolds **2** and **4** using the Cu(I)-catalyzed alkyne-azide cycloaddition (CuAAC).[21,22] We applied the procedure described in the literature to obtain the azido tacrine **5**.[23] For the alkynyl cyclic decapeptides, two distinctly protected decapeptides, in which the arginine residues were protected by Pmc groups (compounds **1** and **3**), were first synthesized as we previously described.[18,19] Introduction of alkynyl groups to decapeptides **1** and **3** was then achieved by coupling propargyloxycarbonyl chloride in a mixture of pyridine/DMF, or 4-pentynoic acid with PyBOP/DIPEA in DMF, on their free lysine residues. In the case of **1**, a large excess of propargyloxycarbonyl chloride was required to observe a total conversion by RP-HPLC. Then the Pmc protecting groups were selectively removed by treatment with TFA to afford the alkynyl building blocks **2** and **4**.

The Cu(I)-catalyzed 1,3-dipolar cycloaddition from templates **2** and **4** was performed with a slight excess of tacrine derivative **5** using copper turnings with a catalytic amount of sodium ascorbate, as we have previously demonstrated the efficiency of this unusual combination.[19] We used a combination of H_2O and CH_3CN that was optimum for the solubility of all precursor



Scheme 1 Synthesis of clicked tacrine conjugates. a) *i*) propargyloxycarbonyl chloride (3.5 eq. per site), pyridine/DMF (1 : 1), 2 h; *ii*) TFA/H_2O (9 : 1), 56% from **1** (2 steps); (b) *i*) 4-pentynoic acid (1.1 eq. per site), PyBOP (1.1 eq. per site), DIPEA (3–4 eq.), DMF (10^{-2} M), 4 h; *ii*) TFA/H_2O (9 : 1), 96% as crude from **3** (2 steps); (c) compound **5** (1.2 eq. per site), copper turnings, sodium ascorbate (0.2 eq.), H_2O/CH_3CN (1 : 1), 40 °C, 6 h, 42%; (d) compound **5** (1.1 eq. per site), copper turnings, sodium ascorbate (0.2 eq.), H_2O/CH_3CN (1 : 1), r.t., 29 h, 27%; (e) 3-methoxy-1-propyne (1.6 eq.), $CuSO_4$ (0.1 eq.), sodium ascorbate (0.2 eq.), H_2O/tBuOH (1 : 2), microwave, 10 min, 42%; (f) and (g), see ref. 18 and 19.

compounds. The conversion of **2**, followed by RP-HPLC, was complete after 6 h and conjugate **6** was isolated in 42% yield after semi-preparative RP-HPLC purification. For conjugate **7**, the reaction time was increased to obtain a total conversion. However **7** was obtained in only 27% isolated yield due to losses during its difficult preparative purification.

The tacrine derivative **8** (Scheme 1), used as a control in thioflavin T assays, was also obtained by a "click reaction" in 42% yield using a classical combination of $CuSO_4$ and sodium ascorbate under microwave irradiation. The cyclopeptide controls **9** and **10**, in which acetyl groups replaced the tacrine subunits, were prepared as described in our previous works.[18,19]

In vitro inhibition studies of $A\beta_{40}$ fibril formation

The ability of conjugates **6** and **7** to inhibit $A\beta_{40}$ fibril formation was studied using thioflavin T (ThT) assays. Results are presented in Fig. 2. The conjugates were co-incubated for 8 days at different concentrations with $A\beta_{40}$ (50 μM) in the presence of ThT (10 μM) and the fluorescence change at 485 nm was measured. ThT was added directly to fibril forming mixture as it has previously been shown to have no effect on the assembly process.[24,25]

Control compounds were also studied in order to compare the activity of conjugates with part of their molecules: the tacrine analogue **8** and cyclodecapeptides **9** and **10** with four or two acetylated lysines respectively (Scheme 1). In order to confirm that the tested compounds would not self-aggregate in the experimental conditions, we also performed ThT assays without $A\beta_{40}$ (data not shown). At a 100 μM concentration, which corresponds to the highest concentration of inhibitors used in the assays, neither conjugate **6** nor conjugate **7** showed ThT binding in the absence of $A\beta_{40}$ after 8 days of incubation. At 100 μM, the tacrine conjugates **6** and **7** significantly reduced ThT fluorescence with an inhibition of 90% and 80% respectively. At this concentration (100 μM),

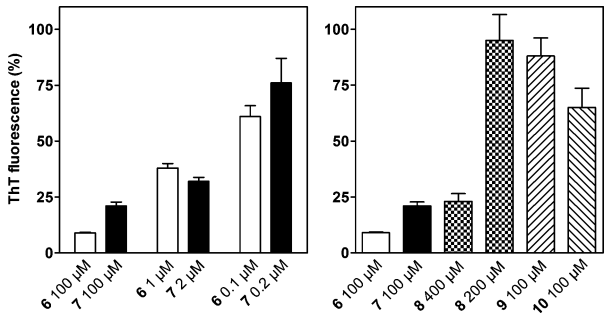


Fig. 2 $A\beta_{40}$ (50 µM) co-incubated for 8 days with (left) conjugates **6** and **7** at the indicated concentrations; (right) conjugates **6** and **7** and the control compounds **8**, **9** and **10** at the indicated concentrations. Values are the maximal fluorescence intensity at 485 nm compared to the control ($A\beta_{40}$ with no inhibitor). Results are the mean ± standard deviation of 2–3 experiments.

only a weak inhibition was observed for the corresponding cyclodecapeptides **9** and **10** without tacrine moieties.[18,19] The inhibitory effect of conjugate **6** can be seen upon comparison with the control tacrine derivative **8**, which is less active at a 4-fold higher concentration (Fig. 2, right). This is in agreement with atomic force microscopy (AFM) observations (Fig. 3). Indeed, when incubating compound **6** at 50 µM no fibril was detected after 12 days of incubation (Fig. 3B) whereas fibrils were observed for tacrine derivative **8** at 200 µM (Fig. 3C). However the fibrils are shorter (0.1–1.5 µm in length) than in the control sample of $A\beta_{40}$ (long protofilaments of 5–10 nm in height and 1–4 µm in length, Fig. 3A) attesting an effect of the tacrine unit on fibril morphology. The cyclodecapeptide **9**, containing four acetylated lysine residues instead of lysinyl-tacrine moieties, showed no inhibitory effect at 100 µM since long fibrils, like those in the control sample, were observed (Fig. 3D).

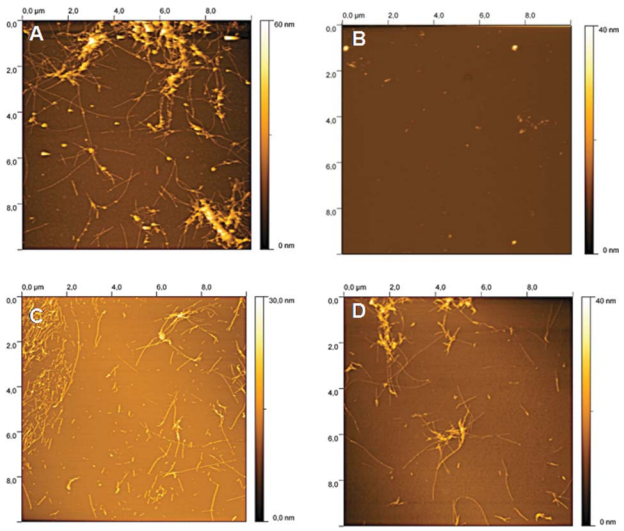


Fig. 3 AFM images (height data) of fibrils formed from $A\beta_{40}$ (50 µM) after 12 days of incubation at 37 °C with the different compounds (scan size 10 µm). A) $A\beta_{40}$ with no inhibitor, B) compound **6** 50 µM, C) compound **8** 200 µM, D) compound **9** 100 µM.

A similar benefit is observed for conjugate **7**, bearing two copies of tacrine, which is up to 4-fold more active at 100 µM (Fig. 2, right) than the tacrine derivative **8** at 200 µM, comparing the moles of tacrine subunits. Moreover, both tacrine conjugates are still active at micromolar concentrations (1 or 2 µM) with an inhibition of more than 50% (Fig. 2, left). For conjugate **6**, additional experiments were performed in an attempt to better understand the inhibitory effect seen with this compound. When performing the ThT assay in the presence of preformed fibrils (seeding experiments), no inhibition of the fibril extension is observed even if a decrease of the polymerization speed was observed. Conjugate **6** is also unable to destabilize preformed $A\beta_{40}$ fibrils (data not shown).

$A\beta_{40}$ fibril binding study

Interaction of compounds **6** and **7** with $A\beta_{40}$ fibrils was investigated by surface plasmon resonance (SPR). Fibrils were made in PBS and immobilized by amine coupling to 5000 RU units on the chip. Binding of the compounds on preformed $A\beta_{40}$ fibrils ($fA\beta_{40}$) were analyzed in graded concentrations at 25 °C for generation of binding curves (Fig. 4).

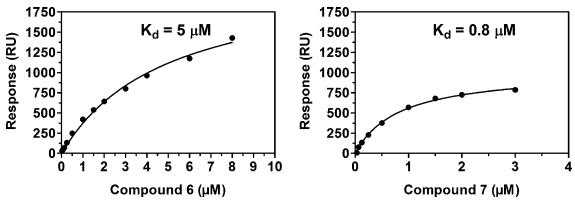


Fig. 4 Adsorption isotherms of the binding of compounds **6** and **7** on $A\beta_{40}$ fibrils.

Compound **7**, containing two tacrine molecules, fitted well to a single-site binding isotherm with an affinity of 0.8 µM. Moreover, the amount of fibrillar bound tacrine conjugates suggests a binding along the fibril at multiple binding sites. Compound **6** bound with a somewhat weaker affinity to fibrils (5 µM) although it contains four tacrine molecules. The plateau level of compound **6** is twice the level of that for compound **7**, which suggests twice the number of binding sites for compound **6**. In analogy to compound **7** the binding curve fitted well to a single-site binding isotherm. Even if compound **6** presents less affinity for $fA\beta_{40}$, it appears that it has a more specific binding than compound **7** as no interaction has been observed with IgM antibody (data not shown). The binding of conjugates **6** and **7** on $fA\beta_{40}$ through their arginine residues was excluded as no effect on the binding was observed by performing competition studies with soluble arginine (data not shown). Finally, conjugates showed no destabilizing activity on $fA\beta_{40}$ (data not shown).

Acetylcholinesterase inhibition study

The capacity of conjugates **6** and **7** to inhibit acetylcholinesterase activity was assessed using the Ellman's method.[26] As shown in Table 1, conjugates **6** and **7** are potent inhibitors of AChE with IC_{50} values in the nanomolar range. No significant difference is obtained for conjugates **6** and **7**, and both are twofold more active than tacrine.

Table 1 *In vitro* evaluation of tacrine conjugates as AChE inhibitors. The IC_{50} values obtained are the average of three independent measurements each realized in triplicate ± SEM

Compounds	IC_{50}/nM
Compound **6**	63 ± 5
Compound **7**	68 ± 10
Tacrine	157 ± 29
	100 ± 2,[27]
	180 ± 20[28]

Discussion

Tacrine was first reported and clinically used as an inhibitor of AChE to improve the cognition in AD patients. Its pharmacological profile has been extended since and a large number of tacrine derivatives have been developed as MTDLs with additional targets than AChE.[5–15] Despite the fact that the lipophilic character of tacrine may be anticipated as an Aβ binding element, only a few reports have investigated the interaction of tacrine derivatives with Aβ. We postulated that in our conjugates **6** and **7** (Fig. 1), the multimeric presentation of tacrine may act as an efficient Aβ recognition domain to target aggregation-prone species and fibrils.

Synthesis

The design of the tacrine conjugates **6** and **7** is based on our previous studies in which we have used arginine-rich cyclodecapeptides as templates to conjugate Aβ ligands and have shown their inhibitory effect toward $Aβ_{40}$ fibril formation compared to the ligands alone.[18,19]

To synthesize the tacrine conjugates, we opted for Cu(I)-mediated alkyne-azide cycloaddition (CuAAC) as the conjugation method between alkynyl peptidic templates bearing two or four arginine residues and an azido tacrine derivative (Scheme 1). The major reason was that CuAAC has already been successfully used by us[19] and others[29–31] to attach various ligands (sugars, small peptides or molecules) onto cyclopeptidic templates. Moreover, the prerequisite alkyne and azide building blocks were synthetically easily accessible based on previously reported works. The modified tacrine molecule with an azide spacer **5** was indeed described in the literature.[23] As for the synthesis of alkynyl peptidic templates **2** and **4**, propargyloxycarbonyl chloride and 4-pentynoic acid were tethered as alkynyl linkers to template precursors **1** and **3**,[18,19] already prepared for other studies. HPLC monitoring of the reactions showed a complete conversion of the precursors **1** and **3**, and after the removal of the Pmc arginine protecting groups, the HPLC analysis of the crudes **2** and **4** revealed in each case only one compound with good purity. Nevertheless, compound **4** appears as a broad peak, probably due to the presence of the four arginine residues. As we have already noticed in our previous work,[19] it could be responsible for the difficulty and loss of material during HPLC purification. For this reason, we preferred to utilize the template **4** directly as a crude compound for its click ligation. The click ligations of the azido tacrine derivative **5** monitored by HPLC were successful from both templates **2** and **4** using an unusual combination of copper turnings with a catalytic amount of sodium ascorbate, which had already proven its efficiency.[19] After HPLC purification, necessary to remove the slight excess of tacrine derivative used for the complete ligation, the conjugates **6** and **7** were obtained in similar yields, 23% and 25% respectively, from the intermediate templates **1** and **3** bearing the free lysine side chains. Taking into consideration the purification difficulties caused by arginine residues, the yields are acceptable for three synthetic steps and are close to those we have reported for similar assemblies.[18,19]

Biological evaluation

We investigated, by ThT and SPR assays, the *in vitro* interaction of **6** and **7** with Aβ. We first performed the ThT assay in order to evaluate the ability of compounds **6** and **7** to interact with the amyloid aggregation process (Fig. 2). To validate this study, the absence of self-aggregation in the assay conditions was verified for both conjugates. The results show that the tacrine conjugates significantly disturb fibril formation from $Aβ_{40}$. Indeed, almost total inhibition is observed for the two conjugates at 100 μM corresponding to a 2 : 1 (compound : $Aβ_{40}$) ratio. At the same ratio, only a weak inhibition is detected for the control templates **9** and **10** without tacrine moieties. Compared to the conjugates, the tacrine monomer **8** is less active at a 4-fold higher ratio of 8 : 1 and totally inactive at a 4 : 1 ratio. Moreover our conjugates remain active even at lower ratios, 0.02 : 1 for **6** and 0.04 : 1 for **7**, with an inhibition higher than 50%. The inhibition is visualized by AFM in which no amyloid fibril is detected for **6**, while fibrils are observed for the monomer **8** at fourfold higher ratio.

Together these results demonstrate that the induced inhibitory activity of conjugates arises from the multimeric presentation of tacrine motifs on the surface of the scaffolds even if we cannot exclude an influence of arginine residues as disrupting elements[32,33] of the aggregation process, especially at high concentrations. Indeed, control template **10**, presenting four arginine residues at a high 2 : 1 ratio with $Aβ_{40}$, has previously revealed by AFM to affect fibril morphology.[19] In addition, this study reveals no significant difference in activity between the two conjugates, especially if we compare the moles of tacrine subunits. In comparison with other previously reported conjugates made from the same arginine-rich templates, conjugates **6** and **7** present an intermediate activity. Whereas a KLVFFA conjugate is more active since almost 100% of inhibition is obtained, at a comparable 0.02 : 1 ratio, quinacrine conjugates are tenfold less active and curcumin conjugates show a comparable inhibition of 40%.[18,19] The β-sheet breaker KLVFFA motif is an Aβ binding domain and it is therefore not surprising that the corresponding conjugate shows a strong activity. Nevertheless the activity of the tacrine conjugates **6** and **7** on Aβ fibril formation is significant compared to well known inhibitors. Even if nordihydroguaiaretic acid (known as NDGA) remains a better inhibitor (50% inhibition at a 0.0028 : 1 ratio),[34] **6** and **7** present better activities than curcumin (inactive at a 0.02 : 1 ratio),[19] peptide inhibitors like LPFFD (active only at a 10 : 1 ratio)[35] or even a four-copy KLVFF dendrimer (90% inhibition of $Aβ_{42}$ aggregation at a 4 : 1 ratio).[36] Compared to tacrine derivatives designed as multipotent anti-Alzheimer drugs, conjugates **6** and **7** appear more effective in preventing self-induced fibril formation than a series of pyrano[3,2-c]-quinoline-6-chlorotacrine (inhibition activities ranging from 12% to 49% for an equimolar concentration with $Aβ_{42}$)[9] or a series of carbazole-6-chlorotacrine ($Aβ_{42}$ self-aggregation inhibition not higher than 36% at a ratio of 0.2 : 1).[14] Indeed, an activity greater than

50% inhibition is obtained for **6** and **7** for a ratio equal to or below 0.04 : 1 (conjugates : $A\beta_{40}$). Nevertheless, we should take into account that, in our case, $A\beta_{40}$ instead of $A\beta_{42}$ was targeted. Even so, in these heterodimers of tacrine as in our conjugates, the combination of several aromatic moieties appears essential for their antiaggregating action.

The Aβ fibril formation process is presumed to occur through a nucleation-dependent polymerization mechanism.[37] The formation of a hydrogen bonding network and hydrophobic interactions are the leading intermolecular forces driving amyloid fibril formation. Inhibitors of fibril formation can act by binding to monomers or to aggregation-prone species during the nucleation phase or by binding to the nascent fibrils during the polymerization phase. In our conjugates, the planar and hydrophobic tacrine units could interact through hydrophobic and π-stacking interactions with misfolded monomers and oligomers during the nucleation phase before they have a chance to form β-sheets. These interactions, associated with the disturbing effect of the arginine scaffolds (through hydrogen bonding and π electron–cation interactions of the guanidinium groups),[33] may block the Aβ association process leading to the nucleus formation. The fact that conjugate **6** is neither able to interfere with the extension phase (seeding experiments) nor able to destabilize preformed fibrils corroborates this hypothesis.

The destabilizing effect of conjugates **6** and **7** on preformed $A\beta_{40}$ fibrils ($fA\beta_{40}$) was also studied by SPR. Neither conjugate **6** nor **7** are able to disassemble preformed fibrils at the surface of the chip, confirming the ThT experiments. Considering that intermediate oligomers in the aggregation process could be more toxic than fibrils, this finding is rather advantageous.

Biosensor assays are also convenient tools for analysing the binding of compounds to Aβ fibrils and conjugates **6** and **7** reveal to highly bind $fA\beta_{40}$. No competition with soluble arginine was observed, suggesting that the interaction with fibrils is not due to the presentation of arginine residues and must involve tacrine moieties. The Langmuir-type (1 : 1) binding curves obtained from the sensorgrams allow the calculation of the dissociation constants with values in the micromolar and submicromolar range (5 and 0.8 μM for conjugates **6** and **7** respectively). The fourfold tacrine conjugate **6** does not show an enhanced binding affinity for $fA\beta_{40}$ compared to the twofold tacrine conjugate **7** as we expected. The presentation of two tacrine ligands seems to be sufficient to efficiently bind fibrils, as shown by affinity values. The steric hindrance of the four ligands in **6** could lead to a less optimal interaction with Aβ fibrils. However the difference in binding of **6** and **7** cannot only be related to the number of tacrine ligands. Indeed, the conjugates differ by the amino acid nature of their scaffold and the spacer arm at the surface of this scaffold. If the high affinity of conjugates for $fA\beta_{40}$ is not related to their ability to inhibit the fibril formation in ThT assays, it constitutes an additional interesting property. Their ability to bind fibrils without disaggregating them may be employed to design new histological markers of β-sheet fibrils in brain tissues. To compare, Congo red, a fluorescent β-sheet indicator, is reported to bind Aβ fibrils at a concentration of 1 μM in similar binding assays.[38] Recently a coumarine-tacrine derivative, first designed as a fluorescent probe to detect cholinesterases inside the AD plaques, revealed by confocal microscopy to bind to amyloid structures rather than to enzymes.[17] The presence of both coumarine and tacrine as aromatic moieties is suggested to contribute to the high affinity of this probe to β-amyloid structures. The multimeric presentation of tacrine ligands in our conjugates could have the same benefit.

Besides their ability to inhibit fibrillogenesis and to bind Aβ fibrils, both conjugates **6** and **7** remain potent AChE inhibitors, exhibiting IC_{50} values in the nanomolar range (63 and 68 nM for conjugates **6** and **7**, respectively). By comparison, the IC_{50} value found for the active site inhibitor tacrine in this study is 157 nM, and is quite similar to those reported in the literature for the electric eel AChE.[27,28] The finding that conjugates retain the activity of the parent tacrine compound with IC_{50} values over twofold lower is interesting, as they are not designed to target the catalytic site of the enzyme. Indeed, the size of our conjugates is not appropriate for interaction with the catalytic site of AChE, which is located at the bottom of a deep and narrow gorge. On the contrary, we can reasonably postulate that our conjugates interact with the peripheral anionic site (PAS) located at the entrance of the gorge near the enzyme's surface. Indeed, it has been shown from investigations of homodimeric tacrine inhibitors[39,40] that tacrine could bind to the PAS through π-stacking interactions with tryptophan, a highly conserved amino acid on the peripheral side of AChE enzymes. The affinity of tacrine for the PAS is known to be low, but the multimeric presentation of tacrine units in our conjugates could be advantageous and responsible for an enhanced binding. The steric hindrance induced by the cyclopeptidic templates at the opening of the active site gorge could then block the access to the substrate. If the inhibitory activity of conjugates **6** and **7** is enhanced compared to the tacrine inhibitor, it is however lower than those reported for bivalent AChE inhibitors designed to simultaneously bind to both peripheral anionic and catalytic sites. With IC_{50} values between 0.81 and 19.7 nM, bis(7)-tacrine derivatives are more potent inhibitors of human recombinant AChE than tacrine alone, for which an IC_{50} value of 424 nM is reported.[7] A similar benefit is obtained for heterodimeric tacrine derivatives which exhibit IC_{50} values in the nanomolar or subnanomolar range for hAChE.[8,9,14,15] The bis(7)-tacrine derivatives and the bivalent ligands mentioned above also reduce AChE-induced Aβ fibrillogenesis as they inhibit the formation of a stable AChE complex with senile plaque components through the peripheral anionic site.[16] Supposed to interact with the PAS of the cholinesterase, our conjugates may act also as inhibitors of AChE-induced Aβ aggregation, although this property has not been evaluated in this study.

Conclusion

Tacrine has already proven successful in the design of multitargeted ligands for combating the multifactorial nature of AD. In addition to its classical anti AChE effect, its activity as tacrine hybrids has been especially proven in AChE-induced Aβ aggregation. This study also highlights its influence on Aβ fibrils when presenting in a multimeric form. In particular, tacrine conjugates prepared herein exhibit a significant ability (i) to inhibit *in vitro* $A\beta_{40}$ self aggregation (ii) to bind $A\beta_{40}$ fibrils and (iii) to retain the AChE inhibitory activity of the parent tacrine with IC_{50} values in the nanomolar range. These results expand the pharmacological profile of tacrine hybrids and may also help to design new chemical probes of Aβ fibrils.

Experimental

General methods and materials

All protected peptides were prepared by solid phase synthesis using Fmoc/tBu strategy on an Advanced ChemTech 348 Ω synthesizer. RP-HPLC analysis and purification were performed on Waters equipment using linear A-B gradients (solvent A: H_2O containing 0.1% trifluoroacetic acid (TFA); solvent B: CH_3CN containing 9.9% H_2O and 0.1% TFA). The analytical column (Nucleosil C18, particle size 3 μm, pore size 120 Å, 30 × 4 mm^2) was operated at 1.3 mL min^{-1} in 15 min run time. Two columns were used in 30 min run time for purification: a preparative Delta-Pak column (C18, particle size 15 μm, pore size 300 Å, 200 × 25 mm^2) at 22 mL min^{-1} and a semi-preparative Nucleosil 100–7 column (C18, particle size 7 μm, pore size 100 Å, 250 × 10 mm^2) at 5 mL min^{-1}. UV monitoring was performed most of the time at 214 and 250 nm. NMR spectra were recorded on a U+ 500 Varian instrument using the residual solvent peak as internal reference. Mass spectra were obtained by electrospray ionization (ESI-MS) on a VG Platform II in positive mode.

Synthesis

The tacrine derivative **5** was synthesized as reported in the literature.[23]

Cyclopeptides **9** and **10** c[K(Ac)RK(Ac)PGK(Ac)RK(Ac)PG] and c[RK(Ac)RPGRK(Ac)RPG] respectively, used as reference compounds, were synthesized as previously described.[18,19]

Cyclodecapeptide **2**. The linear peptide K(Dde)-R(Pmc)-K(Dde)-P-G-K(Dde)-R(Pmc)-K(Dde)-P-G was first built up automatically on Fmoc-Gly-Sasrin® resin (500 mg, 0.69 mmol g^{-1}) and cyclized (530 mg, 2.16 × 10^{-4} mol) in DMF (0.5 mmol L^{-1}) under high dilution using PyBOP (135 mg, 2.59 × 10^{-4} mol) and DIPEA to adjust the pH to 8–9. The white solid powder obtained after precipitation in diethyl ether was solubilised in a solution of 2% hydrazine in DMF to remove Dde protecting groups. The cyclopeptidic intermediate **1** (336 mg, 2.02 × 10^{-4} mol) was then obtained by precipitation in diethyl ether (93% yield from the linear peptide). The cyclopeptide c[KR(Pmc)KPGKR(Pmc)KPG] **1** (38 mg, 1.79 × 10^{-5} mol) was dissolved (0.01 mol L^{-1}) in a mixture of pyridine/DMF (1 : 1). The solution was cooled to 0 °C and propargyloxycarbonyl chloride (0.025 mL, 2.55 × 10^{-4} mol) was added. The solution was stirred for 2 h at r.t. and then concentrated under reduced pressure. The crude product was obtained as a white solid powder after precipitation and washing in diethyl ether. The Pmc removal was realized by dissolving this crude product (25 mg, 1.25 × 10^{-5} mol) in a solution of TFA : H_2O (9 : 1) (0.01 mol L^{-1}). After 2 h, the reaction was concentrated under reduced pressure and the product was precipitated and washed with diethyl ether. After purification by RP-HPLC (C18, 5–100% B in 30 min), compound **2** (17 mg, 1.01 × 10^{-5} mol) was obtained as a white powder in 56% yield. HPLC t_R = 7.4 min. ESI-MS calcd for $C_{66}H_{100}N_{20}O_{18}$ 1460.7; found 1460.7.

Cyclodecapeptide **4**. To a solution of c[R(Pmc)-KR(Pmc)PGR(Pmc)KR(Pmc)PG][19] **3** (100 mg, 4.43 × 10^{-5} mol) in DMF (0.01 mol L^{-1}), were added 4-pentynoic acid (10 mg, 1.01 × 10^{-4} mol), PyBOP (51 mg, 9.81 × 10^{-5} mol) and DIPEA to adjust the pH to 8–9. The reaction was stirred for 4 h at r.t. and then concentrated under reduced pressure. The crude product was dissolved in a solution of TFA : H_2O (9 : 1) (0.01 mol L^{-1}). After 2 h, the reaction was concentrated under reduced pressure and the product was precipitated and washed with diethyl ether. Compound **4** (77 mg, 4.26 × 10^{-5} mol) was obtained as a white powder in 96% yield and was used without further purification. HPLC t_R = 6.5 min. ESI-MS calcd for $C_{60}H_{100}N_{24}O_{12}$ 1348.8; found 1348.6.

Compound **6**. Compound **5** (9.1 mg, 3.41 × 10^{-5} mol) and peptide **2** (12.1 mg, 7.16 × 10^{-6} mol) were dissolved in 750 μL of H_2O : CH_3CN (1 : 1). Cu turnings (200 mg) and (+)-sodium L-ascorbate (0.3 mg, 1.51 × 10^{-6} mol) were added and the mixture was stirred for 6 h at 40 °C. The solution was then filtrated and lyophilised. The residue was precipitated and washed with diethyl ether. Purification by RP-HPLC (C18, 5–100% B in 30 min) was performed to afford conjugate **6** (9.6 mg, 2.99 × 10^{-6} mol) in 42% yield. HPLC t_R = 7.8 min. ESI-MS calcd for $C_{126}H_{168}N_{40}O_{18}$ 2529.3; found 2529.8.

Compound **7**. Compound **5** (7 mg, 2.62 × 10^{-5} mol) and peptide **4** (21 mg, 1.16 × 10^{-5} mol) were dissolved in 988 μL of H_2O : CH_3CN (1 : 1). Cu turnings (100 mg) and (+)-sodium L-ascorbate (24 μL, 0.1 M, 2.4 × 10^{-6} mol) were added and the mixture was stirred for 29 h at r.t. The solution was then filtrated and lyophilised. The residue was precipitated, washed with diethyl ether and purified by RP-HPLC (C18, 15–100% B in 30 min) to afford conjugate **7** (8 mg, 3.12 × 10^{-6} mol) in 27% yield. HPLC t_R = 6.5 min. ESI-MS calcd for $C_{90}H_{134}N_{34}O_{12}$ 1883.1; found 1883.7.

Compound **8**. Compound **5** (20 mg, 7.49 × 10^{-5} mol) and 3-methoxy-1-propyne (10 μL, 1.18 × 10^{-4} mol) were dissolved in 1.5 mL of *t*BuOH : H_2O (2 : 1). (+)-Sodium L-ascorbate (15 μL, 1 M, 1.49 × 10^{-5} mol) and $CuSO_4$ (7.5 μL, 1 M, 7.49 × 10^{-6} mol) were added and the mixture was heated in a domestic microwave for 10 min. The product was purified by RP-HPLC (C18, 10-100% B in 30 min) to afford compound **8** (14.3 mg, 3.17 × 10^{-5} mol) in 42% yield. HPLC t_R = 6.5 min. ESI-MS calcd for $C_{19}H_{23}N_5O$ 337.2; found 336.6. ^{1}H NMR (500 MHz, D_2O): δ(ppm) 1.90 (4H, br s, 2,3-CH_2), 2.56 (2H, br s, 1-CH_2), 2.97 (2H, br s, 4-CH_2), 3.19 (3H, s, CH_3), 4.28 (2H, s, CH_2O), 4.48 (2H, m, CH_2NH), 4.71 (2H, m, CH_2N), 7.59 (1H, br t, *J* 8.5 Hz, 7-H), 7.67 (1H, s, HC═C), 7.72 (1H, br d, *J* 7.0 Hz, 5-H), 7.84 (1H, br t, *J* 8.5 Hz, 6-H), 8.11 (1H, br d, *J* 8.5 Hz, 8-H).

Fibril formation measurement of $A\beta_{40}$

Aggregation of $A\beta_{40}$ was performed in 96-well black polypropylene microplates (Greiner). The aggregation buffer consists of 100 mM sodium phosphate buffer pH 7.4 in which 200 mM of sodium chloride were added. To each well (final volume = 100 μL), 10 μL of the $A\beta_{40}$ peptide stock solution[41] was mixed into 50 μL of the aggregation buffer in the presence of 10 μL of a 100 μM ThT solution giving a final concentration of 50 μM in $A\beta_{40}$ and 10 μM in ThT. Then, 28 μL of water and aliquots of 2 μL of compounds **6**, **7**, **8**, **9** or **10** were added. Compounds were dissolved in DMSO : H_2O (1 : 1) and the final concentrations of DMSO in inhibition studies were less than 2%. Microplates were sealed with a plastic sheet and incubated at 37 °C. Measurements of ThT binding by fluorescence spectroscopy were recorded once or twice daily using a Molecular Devices Spectra MAX Gemini XS microplate spectrophotometer (filters of 445 nm for excitation and 485 nm for emission and a cut-off filter of 475 nm). For imaging

fibrils by atomic force microscopy, we used the same procedure and material as previously described.[19]

$A\beta_{40}$ fibril binding study

All binding experiments were carried out on Biacore 3000 instrument. Fibrils were prepared by incubating $A\beta_{40}$ at 100 μM in PBS at 37 °C with agitation for 24 h. Prior to immobilization the fibrils were briefly sonicated. Fibril surfaces were prepared using standard-amine coupling chemistry.[42] The carboxymethyl dextran surfaces were activated by injecting a 1 : 1 ratio of 0.4 M EDC and 0.1 M NHS for 7 min at a flow rate of 5 μL min^{-1}. $A\beta_{40}$ fibril preparation was diluted to 0.1 mg mL^{-1} in 20 mM sodium acetate, pH 4.6, and injected to the desired immobilized density varied from 4000 to 5000 RU to a CM5 chip. The remaining activated surface groups were blocked by injection of 1 M ethanolamine for 7 min. Analysis of compound binding to fibrils was carried out in duplicate at a concentration range from 0.03 μM to 8 μM with an injection of 100 μL at a flow rate of 50 μL min^{-1}. The experiments were performed in Tris buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.005% surfactant p20, 1 mg mL^{-1} CM-dextran) with 5% DMSO at 25 °C. The chip was regenerated between each injection with 4 M $MgCl_2$. The plateau values of each injection were plotted against the concentration of the compound to generate binding curves. The dissociation constant was determined by fitting the binding curve to a 1 : 1 interaction model.

Acetylcholinesterase inhibition study

Inhibition studies were performed using Ellman's method in 96-well U.V. transparent microplates (Corning®).[26] AChE solution was prepared by dissolving electric eel AChE lyophilized powder (E.C.3.1.1.7, Type V-S, 2000U, Sigma) in 0.1 M phosphate buffer at pH 7.3 to give a 5U enzymatic solution. Solutions of tested inhibitors were prepared in DMSO : H_2O (1 : 1) and concentrations ranged from 0.05 to 1 μM (the final concentrations of DMSO in inhibition studies were less than 2%). Furthermore, 5 mM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) solution and 75 mM ATC (acetylthiocholine) solution were prepared in 0.1 M phosphate buffer with 0.1 M NaCl, pH 7.4. The assay solution consisted of 290 μL of a 0.1 M phosphate buffer pH 8, 5 μL of the 5U AChE solution and 10 μL of the test compound solutions. Then 20 μL of the 5 mM DTNB solution was added, and the reaction was started by addition of 2 μL of the 75 mM ATC substrate solution. The microplate was shaken immediately for 5 s and the absorption at 412 nm was recorded for 5 min. Assays were done with a blank containing all components except AChE in order to account for non-enzymatic reactions. Each concentration of test compounds was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration-inhibition curves.

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Notes and references

1 M. P. Mattson, *Nature*, 2004, **430**, 631–639.
2 A. Rauk, *Chem. Soc. Rev.*, 2009, **38**, 2698–2715.
3 J. Hardy and D. J. Selkoe, *Science*, 2002, **297**, 353–356.
4 A. Lleo, S. M. Greenberg and J. H. Growdon, *Annu. Rev. Med.*, 2006, **57**, 513–533.
5 A. Cavalli, M. L. Bolognesi, S. Capsoni, V. Andrisano, M. Bartolini, E. Margotti, A. Cattaneo, M. Recanatini and C. Melchiorre, *Angew. Chem., Int. Ed.*, 2007, **46**, 3689–3692.
6 D. Munoz-Torrero and P. Camps, *Curr. Med. Chem.*, 2006, **13**, 399–422.
7 M. L. Bolognesi, A. Cavalli, L. Valgimigli, M. Bartolini, M. Rosini, V. Andrisano, M. Recanatini and C. Melchiorre, *J. Med. Chem.*, 2007, **50**, 6446–6449.
8 P. Camps, X. Formosa, C. Galdeano, T. Gomez, D. Munoz-Torrero, M. Scarpellini, E. Viayna, A. Badia, M. V. Clos, A. Camins, M. Pallas, M. Bartolini, F. Mancini, V. Andrisano, J. Estelrich, M. Lizondo, A. Bidon-Chanal and F. J. Luque, *J. Med. Chem.*, 2008, **51**, 3588–3598.
9 P. Camps, X. Formosa, C. Galdeano, D. Munoz-Torrero, L. Ramirez, E. Gomez, N. Isambert, R. Lavilla, A. Badia, M. V. Clos, M. Bartolini, F. Mancini, V. Andrisano, M. P. Arce, M. I. Rodriguez-Franco, O. Huertas, T. Dafni and F. J. Luque, *J. Med. Chem.*, 2009, **52**, 5365–5379.
10 D. M. Du and P. R. Carlier, *Curr. Pharm. Des.*, 2004, **10**, 3141–3156.
11 P. W. Elsinghorst, J. S. Cieslik, K. Mohr, C. Trankle and M. Gutschow, *J. Med. Chem.*, 2007, **50**, 5685–5695.
12 H. Fu, W. Li, J. Luo, N. T. Lee, M. Li, K. W. Tsim, Y. Pang, M. B. Youdim and Y. Han, *Biochem. Biophys. Res. Commun.*, 2008, **366**, 631–636.
13 M. Rosini, V. Andrisano, M. Bartolini, M. L. Bolognesi, P. Hrelia, A. Minarini, A. Tarozzi and C. Melchiorre, *J. Med. Chem.*, 2005, **48**, 360–363.
14 M. Rosini, E. Simoni, M. Bartolini, A. Cavalli, L. Ceccarini, N. Pascu, D. W. McClymont, A. Tarozzi, M. L. Bolognesi, A. Minarini, V. Tumiatti, V. Andrisano, I. R. Mellor and C. Melchiorre, *J. Med. Chem.*, 2008, **51**, 4381–4384.
15 M. I. Fernandez-Bachiller, C. Perez, G. C. Gonzalez-Munoz, S. Conde, M. G. Lopez, M. Villarroya, A. G. Garcia and M. I. Rodriguez-Franco, *J. Med. Chem.*, 2010, **53**, 4927–4937.
16 G. V. De Ferrari, M. A. Canales, I. Shin, L. M. Weiner, I. Silman and N. C. Inestrosa, *Biochemistry*, 2001, **40**, 10447–10457.
17 P. W. Elsinghorst, W. Hartig, S. Goldhammer, J. Grosche and M. Gutschow, *Org. Biomol. Chem.*, 2009, **7**, 3940–3946.
18 G. T. Dolphin, S. Chierici, M. Ouberai, P. Dumy and J. Garcia, *ChemBioChem*, 2008, **9**, 952–963.
19 M. Ouberai, P. Dumy, S. Chierici and J. Garcia, *Bioconjugate Chem.*, 2009, **20**, 2123–2132.
20 Y. Porat, A. Abramowitz and E. Gazit, *Chem. Biol. Drug Des.*, 2006, **67**, 27–37.
21 M. Meldal and C. W. Tornoe, *Chem. Rev.*, 2008, **108**, 2952–3015.
22 M. G. Finn and V. V. Fokin, *Chem. Soc. Rev.*, 2010, **39**, 1231–1232.
23 W. G. Lewis, L. G. Green, F. Grynszpan, Z. Radic, P. R. Carlier, P. Taylor, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 1053–1057.
24 R. J. Chalifour, R. W. McLaughlin, L. Lavoie, C. Morissette, N. Tremblay, M. Boule, P. Sarazin, D. Stea, D. Lacombe, P. Tremblay and F. Gervais, *J. Biol. Chem.*, 2003, **278**, 34874–34881.
25 H. LeVine, 3rd, *Protein Sci.*, 1993, **2**, 404–410.
26 G. L. Ellman, K. D. Courtney, V. Andres, Jr. and R. M. Feather-Stone, *Biochem. Pharmacol.*, 1961, **7**, 88–95.
27 L. Pan, J. H. Tan, J. Q. Hou, S. L. Huang, L. Q. Gu and Z. S. Huang, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 3790–3793.
28 J. Marco-Contelles, R. Leon, C. de Los Rios, A. Guglietta, J. Terencio, M. G. Lopez, A. G. Garcia and M. Villarroya, *J. Med. Chem.*, 2006, **49**, 7607–7610.
29 M. Galibert, P. Dumy and D. Boturyn, *Angew. Chem., Int. Ed.*, 2009, **48**, 2576–2579.
30 J. Wang, H. Li, G. Zou and L. X. Wang, *Org. Biomol. Chem.*, 2007, **5**, 1529–1540.
31 O. Avrutina, M. Empting, S. Fabritz, M. Daneschdar, H. Frauendorf, U. Diederichsen and H. Kolmar, *Org. Biomol. Chem.*, 2009, **7**, 4177–4185.
32 T. J. Gibson and R. M. Murphy, *Biochemistry*, 2005, **44**, 8898–8907.
33 T. Arakawa and K. Tsumoto, *Biochem. Biophys. Res. Commun.*, 2003, **304**, 148–152.

34 K. Ono, Y. Yoshiike, A. Takashima, K. Hasegawa, H. Naiki and M. Yamada, *J. Neurochem.*, 2003, **87**, 172–181.
35 C. Soto, M. S. Kindy, M. Baumann and B. Frangione, *Biochem. Biophys. Res. Commun.*, 1996, **226**, 672–680.
36 S. M. Chafekar, H. Malda, M. Merkx, E. W. Meijer, D. Viertl, H. A. Lashuel, F. Baas and W. Scheper, *ChemBioChem*, 2007, **8**, 1857–1864.
37 F. Chiti and C. M. Dobson, *Annu. Rev. Biochem.*, 2006, **75**, 333–366.
38 M. J. Cannon, A. D. Williams, R. Wetzel and D. G. Myszka, *Anal. Biochem.*, 2004, **328**, 67–75.
39 E. H. Rydberg, B. Brumshtein, H. M. Greenblatt, D. M. Wong, D. Shaya, L. D. Williams, P. R. Carlier, Y. P. Pang, I. Silman and J. L. Sussman, *J. Med. Chem.*, 2006, **49**, 5491–5500.
40 Y. P. Pang, P. Quiram, T. Jelacic, F. Hong and S. Brimijoin, *J. Biol. Chem.*, 1996, **271**, 23646–23649.
41 G. T. Dolphin, M. Ouberai, P. Dumy and J. Garcia, *ChemMedChem*, 2007, **2**, 1613–1623.
42 C. W. Cairo, A. Strzelec, R. M. Murphy and L. L. Kiessling, *Biochemistry*, 2002, **41**, 8620–8629.