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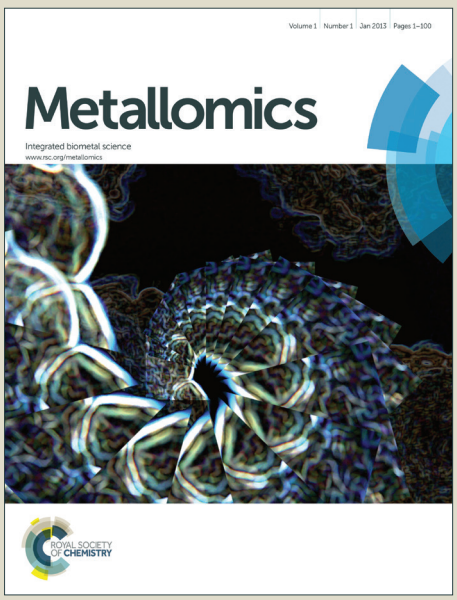

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Neuroprotective Peptide-Macrocycle Conjugates Reveal Complex Structure-Activity Relationships in their Interactions with Amyloid β

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Abstract

Interactions between amyloid β (Aβ) and metal ions are thought to mediate the neuropathogenic effects of Aβ in Alzheimer's disease. The construction of small molecules capable of synergistically chelating metal ions and recognizing Aβ would allow new insights into the biology of this disease and provide a possible therapeutic approach. We report herein the synthesis and biological evaluation of tetraazamacrocycle-(G)KLVFF hybrids and their metal complexes. The results obtained from ThT and bis-ANS extrinsic fluorescence assays, tyrosine intrinsic fluorescence assay and proteolytic assay imply complex, multifaceted structure-activity relationships in the interaction of these conjugates with Aβ. Many of the compounds tested rescued cells from Aβ-induced cytotoxicity. The attendant simplicity and ready diversification of the synthesis of these conjugates makes them attractive for further investigation.

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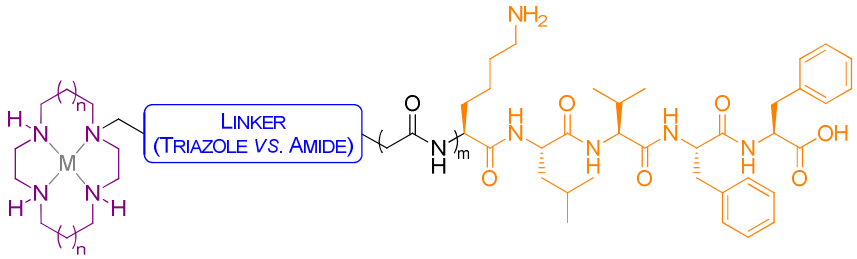
Novel neuroprotective peptide-macrocycle conjugates exhibit complex, multifaceted structure-activity relationships in their interactions with amyloid β. The attendant simplicity and ready diversification of their synthesis makes them a promising class of compounds for further investigation.

Graphical Abstract



COMPLEX SARS IN THE INTERACTION WITH Aβ

Introduction

Alzheimer's disease (AD) is a progressive and multifactorial neurodegenerative disorder leading to the most common form of dementia in the elderly.[1,2] The disease imposes a huge and growing burden on society.[3] Progress towards understanding the underlying cause of AD has been made across a number of disciplines, yet its etiology and pathogenesis remain to be fully and precisely elucidated.[4] Though it is not without its critics,[5-8] the widely-supported amyloid hypothesis posits that aggregation of amyloid β (Aβ) and subsequent deposition into senile plaques (SPs) are involved in the progression of AD.[9]

Metal ions play an important role in the assembly of Aβ.[1,10] Zinc(II)[11] and copper(II),[12] even at the trace (nanomolar) concentrations found in commonly-used laboratory buffers and culture media,[13] induced marked Aβ aggregation *in vitro*. Elevated concentrations of copper, zinc and iron have been observed in SPs of AD patients.[14] Redox-active copper(I/II) and iron(II/III) bound to Aβ can undergo Fenton-type chemistry to generate reactive oxygen species such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH),[15,16] which can cause considerable oxidative damage to biological molecules and trigger neurodegeneration. These findings suggest that metal chelators could offer interesting new therapeutic benefits for AD by disrupting metal-Aβ interactions.

Current research efforts in this area centre on the construction of small molecules capable of synergistically chelating metal ions and recognizing Aβ.[17,18] Such molecules consist of a metal chelator (*e.g.* clioquinol) and a known Aβ recognition group (*e.g.* curcumin, thioflavin-T). A particularly intriguing example of such a bifunctional molecule is the cyclen-pentapeptide hybrid **1** (Figure 1A) in which cyclen is the metal chelator and the KLVFF peptide the Aβ recognition moiety.[19] This hybrid was found to capture copper(II) bound to Aβ, become proteolytically active, interfere with Aβ oligomerization and aggregation, cleave Aβ into fragments, and prevent H_2O_2 formation and toxicity in neuronal cell culture. This precedent raises two interesting questions: (1) Is sequestration of the metal ion pivotal? (2) What structural elements are required for proteolysis to occur? Answering these questions may help to explain, among other things, why molecule **1** does not self-cleave. We became interested in exploring this

structure for its possible therapeutic potential but also more broadly for the insights it might give to the behaviour of Aβ. We report herein the synthesis and biological evaluation of ten novel tetraazamacrocycle-(G)KLVFF hybrids: the amide- or triazole-linked derivatives **2** and **4**–**6** (Figure 1A) and their metal complexes **20**, **21** and **23**–**26** (Scheme 1).

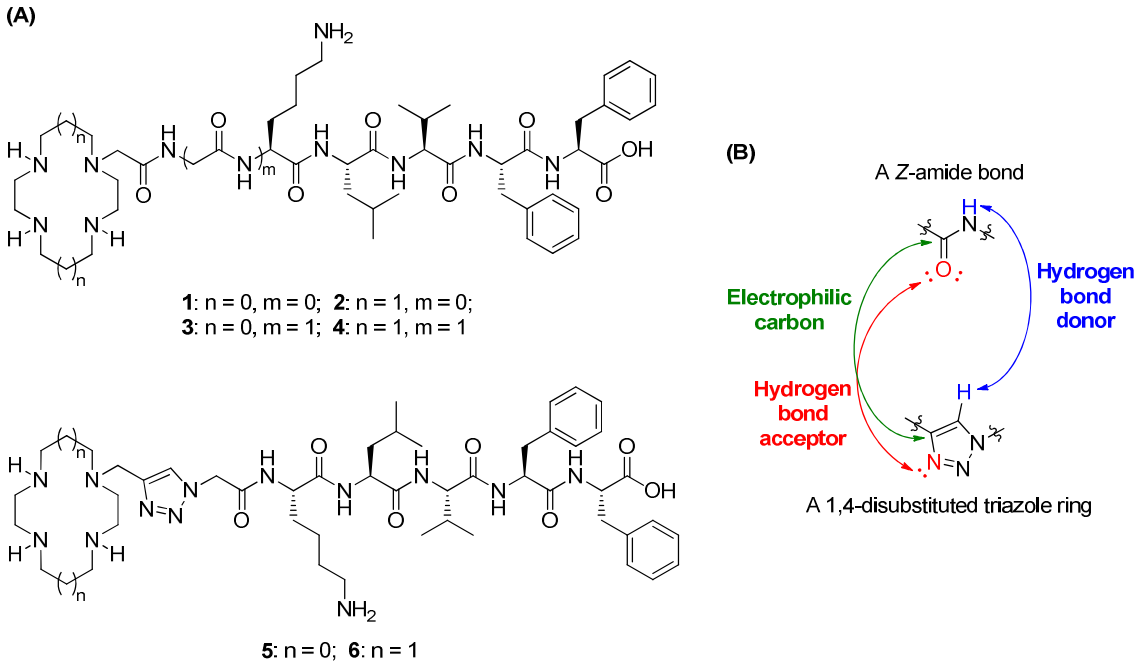


Figure 1. (A) Structures of tetraazamacrocycle-(G)KLVFF hybrids **1**–**6**; (B) Electronic similarities between a *Z*-amide bond and a 1,4-disubstituted triazole ring.[20]

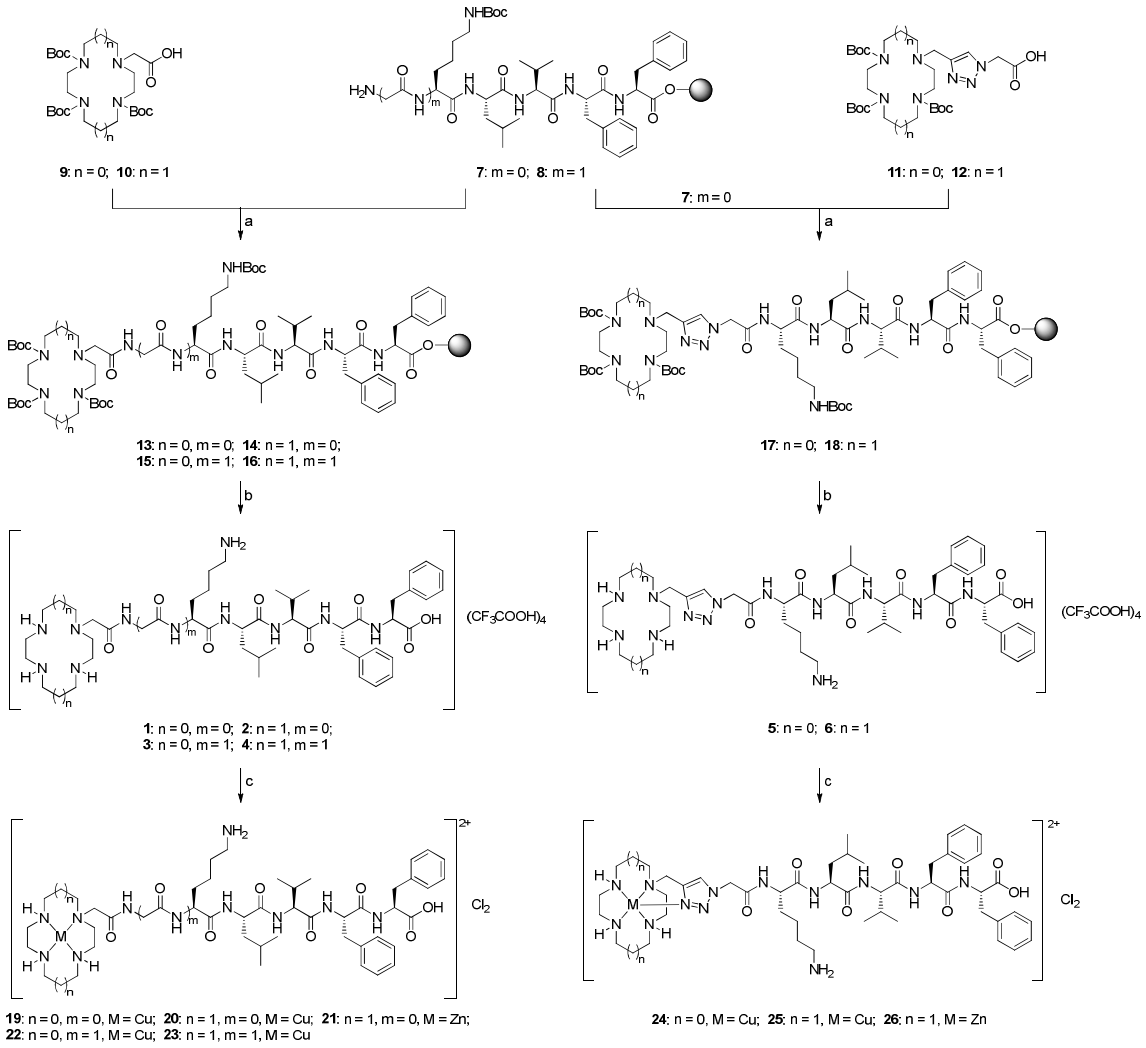
These molecules were designed to provide insight into the structure-activity relationships that might be operating. Cyclam has a stronger binding affinity for copper(II) than does cyclen (log K: 26.5 for copper(II)-cyclam *vs.* 23.3 for copper(II)-cyclen),[21] suggesting that copper(II)-cyclam complexes are more likely to be formed *in vivo* than the corresponding copper(II)-cyclen complexes.[22] Furthermore, the metal complexes of cyclen and cyclam tend to have different hydrolytic abilities, thus cyclam-KLVFF hybrid **2** was chosen to evaluate the role of the azamacrocyclic metal complex in any observed peptide cleavage. Synthetic pentapeptide KLVFF, a short Aβ fragment ($A\beta_{16\text{-}20}$), has been shown to bind full-length Aβ and disrupt its assembly into Aβ fibrils.[23,24] To examine the importance of the length and nature of the recognition sequence, and its position relative to the metal centre, additional glycine spacers were introduced (hybrids **3** and **4**). The triazole moiety was incorporated into

tetraazamacrocycle-KLVFF hybrids to explore whether coordinative saturation of copper(II) with the additional triazole ligand affects proteolytic activity (hybrids **5** and **6**). Given that hybrids **5** *vs.* **3** and **6** *vs.* **4** are structurally identical except for the linker (triazole *vs.* amide), the biological potential of 1,4-disubstituted triazole as a *Z*-amide bioisostere[20] (Figure 1B) could be assessed by comparing triazole-linked hybrids **5** and **6** with amide-tethered hybrids **3** and **4**. All six tetraazamacrocycle-(G)KLVFF hybrids **1**–**6** and their corresponding copper(II) and zinc(II) complexes were synthesized and evaluated; the change in metal ion is of obvious interest to determine whether the specific nature of the metal complex is important in the biological potency of these conjugates.

Results and Discussion

(a) Synthesis of Tetraazamacrocycle-(G)KLVFF Hybrids and their Metal Complexes

The resin-bound oligopeptides KLVFF **7** and GKLVFF **8** were assembled *via* Fmoc-strategy solid phase peptide synthesis (SPPS) on Wang resin (Scheme S1, Supporting Information), and further coupled with tri-Boc-tetraazamacrocycle-acetic acid **9** or **10** to give resin-bound Boc-protected tetraazamacrocycle-(G)KLVFF compounds **13**–**16** (Scheme 1). Removal of Boc groups and detachment from the solid support were achieved in one pot by treatment of resin-bound compounds **13**–**16** with a cleavage/scavenger cocktail of TFA/TIS/H_2O (90:5:5). The crude products were purified by RP-HPLC and lyophilized to give the desired amide-tethered hybrids **1**–**4** as fluffy white solids in good overall yields. This solid-phase based synthetic procedure was successfully applied to the preparation of triazole-linked hybrids **5** and **6** by introducing tri-Boc-tetraazamacrocycle-triazole-acetic acid **11** and **12** respectively to the resin-bound oligopeptide **7**. Elemental analysis data indicated that each of the isolated compounds **1**–**6** incorporated four equivalents of constitutive TFA (presumably three associated with secondary amino groups of the tetraazamacrocycle and one with the ε-amino group of the lysine residue).



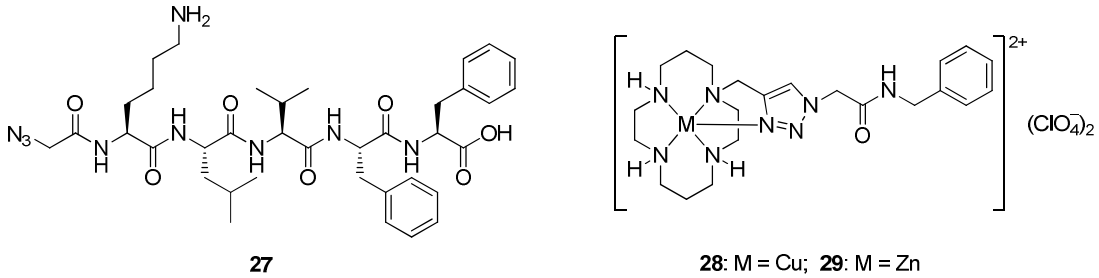
Scheme 1. Synthesis of tetraazamacrocycle-(G)KLVFF hybrids **1**–**6** and their metal complexes **19–26**. Reagents and conditions: (a) appropriate carboxylic acid (**9**, **10**, **11** or **12**), PyBOP, NMM, DMF, rt, 1 h; (b) TFA/TIS/H_2O (90:5:5), rt, 2 h, followed by RP-HPLC purification, **1**: 53%, **2**: 63%, **3**: 52%, **4**: 60%, **5**: 60%, **6**: 58%; (c) $CuCl_2 \cdot 2H_2O$ or $ZnCl_2$, EtOH, reflux, 6 h, **19**: 94%, **20**: 81%, **21**: 54%, **22**: 85%, **23**: 88%, **24**: 67%, **25**: 53%, **26**: 69%.

The trifluoroacetate salts of compounds **1**–**6** were complexed directly with copper(II) and zinc(II) as reported previously for related systems.[25-27] Reaction with $CuCl_2 \cdot 2H_2O$ was carried out in EtOH at reflux for 6 hours to afford copper(II)-tetraazamacrocycle complexes **19**, **20** and **22**–**25**, which were isolated by centrifugation. The copper(II)-cyclen complexes appear blue whereas the copper(II)-cyclam complexes are purple powders (Figure S1, Supporting Information). Zinc(II) complexes of hybrids **2** and **6** were also prepared by reacting the ligands with $ZnCl_2$ under similar conditions. Other metal cyclam-amino acid/peptide complexes that we have reported previously all exhibited characteristic singly charged cations in the high resolution mass spectra.[25] In

contrast, all the metal complexes of tetraazamacrocycle-(G)KLVFF hybrids gave rise exclusively to a cluster of $[M\text{-}2Cl]^{2+}$ peaks with the correct isotope patterns. The purity of these metal complexes was confirmed by elemental analysis (Supporting Information). The UV-Vis spectra of the copper(II) complexes showed that λ_{max} and ε values associated with the cyclen complexes (λ_{max} = 582–591 nm, ε = 211–258) were larger than those for the corresponding cyclam complexes (λ_{max} = 552–555 nm, ε = 110–138). Absolute values of λ_{max} and ε are expected to be solvent-, pH- and substituent-dependent, but the same relationship of these values (cyclen > cyclam) has been observed previously,[28] as has the reverse relationship (cyclam > cyclen).[29] No significant differences in λ_{max} and ε values were observed between the triazole-linked complexes and the amide-tethered complexes.

(b) *In Vitro* Biological Evaluation

With tetraazamacrocycle-(G)KLVFF hybrids **1**–**6** and their metal complexes **19**–**26** in hand, a series of *in vitro* biological assays was conducted using $A\beta_{42}$ that included thioflavin-T (ThT) extrinsic fluorescence, tyrosine intrinsic fluorescence, bis-ANS extrinsic fluorescence and neurotoxicity assays, along with MALDI-TOF-MS analysis. Azide-capped pentapeptide **27** (see Scheme S1, Supporting Information for the synthetic procedure) and the simple, non-peptidic metal-cyclam complexes **28** and **29**[25] were also evaluated as controls (Figure 2). The fresh Aβ stock solution used for these studies was prepared by a modified literature procedure (Supporting Information).[19] Pretreatment of Aβ with 1,1,1,3,3,3-hexafluoroisopropanol has been previously considered to be of benefit with respect to solubilizing the peptide and monomerizing β-sheet peptide aggregates, however this step was omitted because the alcohol has recently been shown to increase Aβ aggregation in solution.[30]



27 **28**: M = Cu; **29**: M = Zn

Figure 2. Structures of three control compounds **27**–**29**.

(i) ThT Extrinsic Fluorescence Assay

The fluorescence emission maximum of ThT at 480–490 nm (excitation at 440–450 nm) is dramatically enhanced by its binding to Aβ fibrils.[31,32] Accordingly, the fluorescence of ThT is widely used to quantify the inhibition of Aβ fibril formation in the presence of anti-amyloidogenic compounds *in vitro*.

A continuous ThT extrinsic fluorescence assay[33,34] was employed to determine the effects of compounds **1**–**6** and **19**–**29** on Aβ aggregation. These compounds (10 and 50 μM) were incubated with monomeric Aβ (5 μM) and ThT (20 μM) in PBS buffer (pH 7.4) for 23 hours, and the fluorescence of ThT was measured continuously throughout the incubation (excitation at 444 nm and emission at 485 nm). It was found that addition of the test compounds gave rise to (1) an increase (**1**, **3**, **27** and **29**), (2) a decrease (**2**, **6**, **20**, **21**, **23**, **24** and **26**) or (3) little change (**4**, **5**, **19**, **22**, **25** and **28**) in the fluorescence intensity with comparison to that obtained from the case where monomeric Aβ alone was incubated with ThT (Figure 3 and Figure S2, Supporting Information), demonstrating that these compounds could (1) promote, (2) inhibit or (3) hardly affect the Aβ fibril formation respectively (Table 1).

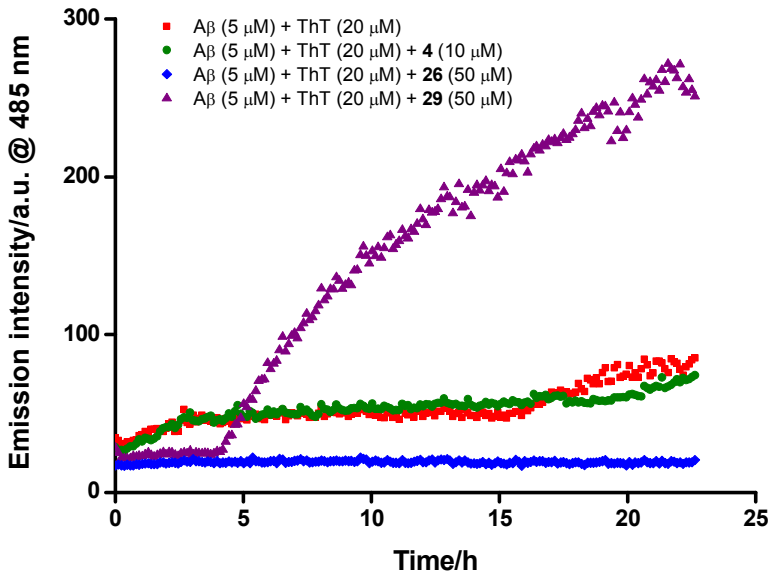


Figure 3. Selected data from ThT extrinsic fluorescence assay: addition of the peptidic zinc(II) complex **26** (50 μM) quenched the ThT fluorescence (blue diamond) with comparison to that obtained from an incubated solution of Aβ (5 μM) and ThT (20 μM) alone (red square), but the simple, non-peptidic zinc(II) complex **29** (50 μM) enhanced the ThT fluorescence (purple up-pointing triangle). The free ligand **4** (10 μM) exerted little influence on the ThT fluorescence (green circle).

Table 1. Effects of compounds **1**–**6** and **19**–**29** at concentrations of 10 and 50 μM on Aβ aggregation.

Compound	Effect on Aβ Aggregation*	
	10 μM	50 μM
1	0	+++
2	−−−	0
3	+++	0
4	0	0
5	0	0
6	−	0
19	−	−
20	0	−−−
21	−	−−−
22	0	0
23	0	−−
24	0	−−
25	0	0
26	−	−−−
27	0	++
28	0	0
29	+	+++

* (1) Inhibition: weak (−), moderate (−−), strong (−−−); (2) No effect: 0; (3) Promotion: weak (+), moderate (++), strong (+++).

Zinc(II) complexes **21** and **26** strongly inhibited formation of Aβ fibrils at both concentrations. The equivalent copper(II) complexes **20** and **25** respectively showed significantly reduced inhibitory activity: some activity was observed for **20** but only at high concentration, while no activity was observed for **25** at both concentrations. This result implies that the nature of the metal ion in such conjugates is an important factor in Aβ fibril inhibitory activity. The corresponding free ligands **2** and **6** had little effect on Aβ fibril formation at 50 μM, but surprisingly exhibited inhibitory activity at 10 μM. Copper(II) complexes **23** and **24** displayed modest activity against Aβ fibril formation at high concentration, while the corresponding free ligands **4** and **5** exerted little influence on Aβ aggregation. As ThT fluorescence is a spectroscopic measure of aggregation, and since it is known that the addition of compounds that are spectroscopically active can

skew the results,[35] we confirmed these results using a pelleting assay, where the amount of aggregate formed over 24 hours was measured by the proportion of Aβ peptide that pelleted at 100000 × *g* (Figure S3). The results showed that the ThT assay was measuring the proportion of aggregated Aβ accurately, confirming the results presented in Table 1. In addition, aggregation could be influenced by an interaction between the phosphate buffer and metal ions. We find no difference in the results when the PBS buffer is substituted for Tris-HCl (20 mM, pH 7.5), suggesting that the PBS buffer is not interfering with the aggregation assay.

The copper(II)-cyclen complexes **19** and **22** had been reported to inhibit markedly the formation of ThT-positive Aβ aggregates.[19] However, in the present study, these two complexes were found to have little effect on Aβ aggregation at either 10 or 50 μM. The corresponding free amine ligands **1** and **3**, which differ only in the length of peptide sequences (KLVFF in **1** *vs.* GKLVFF in **3**), showed contrasting results: hybrid **1** significantly promoted the formation of Aβ fibrils at high concentration (but did not affect Aβ aggregation at low concentration), whereas compound **3** strongly activated Aβ fibril generation at low concentration (but had little effect on Aβ fibril formation at high concentration).

The peptide control **27** moderately accelerated Aβ fibril formation at high concentration. The simple (non-peptidic) zinc(II) complex **29** promoted the generation of Aβ fibril. In contrast, the equivalent copper(II) complex **28** showed no activity against Aβ fibril formation, providing further evidence for the importance of the nature of the metal ion.

The promotion of Aβ aggregation by compounds **1**, **3**, **27** and **29** was signalled by a dramatic increase in ThT fluorescence intensity during the incubation. It is conceivable that these compounds may somehow themselves aggregate to give a false positive, however incubation of each of compounds **1**, **3**, **27** and **29** with ThT in the absence of Aβ did not trigger any measurable fluorescence change (this control was performed for every compound used in this study, including the zinc(II) and copper(II) complexes, with the same result). This result confirmed that these compounds on their own do not form ThT-positive aggregates. There remains the possibility that ThT-positive

heterofibrils are being formed between the added compounds and Aβ, which from a biochemical standpoint warrants further investigation.

These data reveal few if any obvious trends, making it difficult to develop a unified rationale for the effects observed. Overall, the peptidic zinc(II) complexes **21** and **26** exhibited the strongest Aβ fibril inhibitory activity among the test compounds whereas the simple, non-peptidic zinc(II) complex **29** was the only compound to accelerate Aβ fibril formation at both concentrations. None of the copper(II) complexes (**19**, **20**, **22**–**25** and **28**) promoted Aβ aggregation. Two of the unmetallated ligands (**2** and **6**) showed inhibitory activity against Aβ fibril formation at low concentration but hardly affected Aβ aggregation at high concentration, whereas the rest of them (**1** and **3**–**5**) either exerted little influence on or even strongly accelerated the formation of Aβ fibrils. There are no obvious patterns when comparing the different chelators (cyclam *vs.* cyclen), different spacers (*N*-benzylamide *vs.* KLVFF *vs.* GKLVFF), and different linkers (triazole *vs.* amide). The conclusion from this assay is that, rather than a simple Aβ:(G)KLVFF interaction giving a pre-defined outcome, there must be a complex combination of factors that control the interaction between these compounds and Aβ. It is possible that heterofibril assemblies[36,37] form between the compounds and Aβ under the control of complex noncovalent interactions; while this is not equivalent to inhibiting fibril formation, such assemblies could still be important from a medicinal chemistry perspective. Further work is required to investigate this possibility.

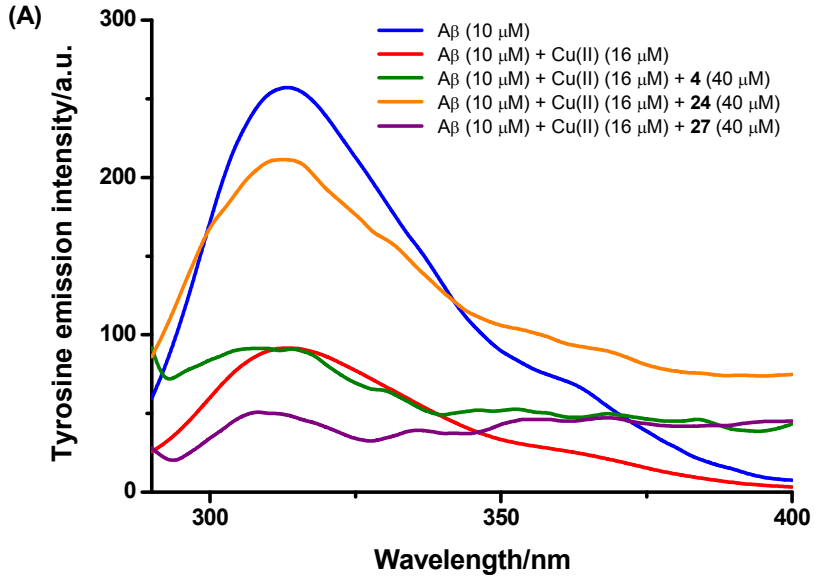
(ii) MALDI-TOF-MS

Given the established proteolytic activity of metal-cyclen complexes,[19,38,39] MALDI-TOF-MS was carried out to evaluate the ability of the metal complexes **19**–**26** to cleave Aβ species; the free ligands **1**–**6** were also evaluated for comparison. Each compound was incubated with Aβ in PBS buffer (pH 7.4) for 4 and 7 days, and the resulting mixture desalted and analyzed by MALDI-TOF-MS. In all cases, the mass spectra showed no evidence for Aβ cleavage, suggesting that none of these compounds promote Aβ cleavage under the tested conditions. Metal complexes **19** and **22** had previously been reported to cleave Aβ species under similar conditions,[19] but this was not observed in this study (Figure S4, Supporting Information).

(iii) Tyrosine Intrinsic Fluorescence Assay

Tyrosine intrinsic fluorescence of Aβ is quenched when copper(II) binds to the peptide and regained by addition of copper(II) chelators.[19,40-42] This assay was used to explore whether tetraazamacrocycle-(G)KLVFF hybrids **1**–**6** are capable of capturing copper(II) bound to Aβ. The corresponding metal complexes **19**–**26**, azide-capped pentapeptide **27** and the two simple metal-cyclam complexes **28** and **29** served as controls.

Excitation of Aβ (10 μM) in Tris buffer (50 mM, pH 7.5) at 275 nm gave rise to the expected time-invariant maximum emission intensity at *ca.* 314 nm (Figure 4A). This tyrosine fluorescence was quenched upon addition of copper(II) chloride (16 μM) as previously reported,[40,42] and subsequent incubation for 1 hour did not cause any further decrease (Figure S5, Supporting Information). After the 1-hour incubation of Aβ with copper(II), the test compound (40 μM) was added and co-incubated for a further hour. The total fluorescence of these three-component mixtures was measured, from which any intrinsic fluorescence of the test compounds themselves (measured separately – Figure S5, Supporting Information) was subtracted. The resulting fluorescence intensity was compared with that obtained from the incubated mixture of Aβ and copper(II) alone to determine whether the test compounds could extract and capture copper(II) bound to Aβ (Figure 4). Alternate additions of these compounds, either preloading the compounds with copper(II), or pre-incubating the Aβ with the compounds did not alter the results.



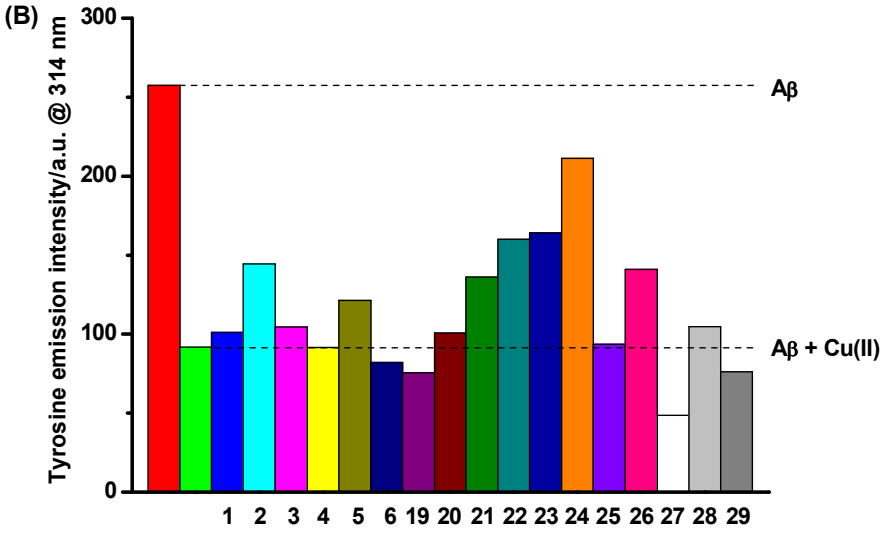


Figure 4. (A) Selected data from tyrosine intrinsic fluorescence assay: tyrosine emission intensity of Aβ (10 μM) in Tris buffer (50 mM, pH 7.5) at *ca.* 314 nm (blue) was quenched upon addition of copper(II) chloride (16 μM) (red); subsequent incubation of the resulting mixture with compound **4**, **24**, or **27** (40 μM) had little effect on (green), partially restored (orange), or further reduced (purple) the tyrosine intrinsic fluorescence respectively; (B) Summary of the effects of compounds **1**-**6** and **19**–**29** on the copper(II)-induced quenching of Aβ tyrosine fluorescence.

Hybrid **1** had previously been reported to reverse the copper(II)-induced quenching of Aβ tyrosine fluorescence due to sequestration of the metal ion from Aβ.[19] However, in the present study, this free ligand exerted little effect on the tyrosine fluorescence of copper(II)-bound Aβ (Figure 4B). Similar results were observed for the ligands **3**, **4** and **6**. In contrast, addition of ligands **2** and **5** resulted in the partial recovery of the tyrosine fluorescence, indicating a low to moderate ability to sequester copper(II) from Aβ.

Surprisingly, the tyrosine intrinsic fluorescence was partially restored upon incubation with several of the metal complexes (**21**–**24** and **26**). These compounds lack any obvious metal-chelating ability: the azamacrocyle already holds a metal ion, and the peptide portion shows no demonstrable ability to sequester copper(II) under these conditions when tested as compound **27**. These results therefore imply that beyond (or perhaps instead of) the chelation effect, another, different interaction contributes to the revival of tyrosine fluorescence. The other compounds tested exert little or no influence on the tyrosine fluorescence. Compound **27** in fact further reduced the tyrosine fluorescence, which may be due to the oxidation of the tyrosine residue by the azido group.[43] As many of these compounds are coloured we cannot rule out some indirect influence on the tyrosine fluorescence through inner-filtering effects. Three points in

our results suggest that this effect is not a significant factor: i) the concentration of the compound, while fairly high, does not give rise to an OD greater than 0.1 arbitrary units at the excitation wavelength of tyrosine; ii) only one uncoloured compound decreased the tyrosine fluorescence further, which, if inner filtering were significant, should have happened with more of the coloured compounds; and iii) mixtures of Aβ and the compounds in the absence of copper(II) did not indicate any significant effect of either molecule on the fluorescence signal of the other, i.e. the total tyrosine fluorescence was simply the sum of the respective signals from the compound and Aβ.

Overall, more of the metal complexes were capable of restoring the tyrosine fluorescence than the free ligands, suggesting that alternative interaction(s) between metal complexes and copper(II)-bound Aβ (*i.e.* other than the chelation and sequestration of copper), are responsible for this process. Thus we propose that the fluorescence is regained not through the chelation of copper, but possibly *via* the displacement of copper(II) from Aβ by the binding of these compounds. Previous deployment of this assay has used either glycine or histidine in large excess to bind copper[40,42] rather than a discrete macrocyclic chelator to compete with Aβ for copper(II) as used here; it is plausible that in those cases too a binding interaction or displacement is occurring, rather than simple metal chelation as previously proposed. While the tyrosine fluorescence results observed in the present study would be explained by a more complex interaction between Aβ and the compounds tested, more work is required to validate this hypothesis. Such work is clearly important to confirm the meaning of this assay more generally.

(iv) Bis-ANS Extrinsic Fluorescence Assay

The ability of compounds **1**–**6** and **19**–**29** to interfere with zinc(II) related enhancement of Aβ self-association was analyzed using the dye 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS). This dye has been shown to be particularly useful for the analysis of metal-Aβ interactions:[44-46] the addition of a metal ion (zinc(II) in particular) causes a very large and persistent increase in the intensity of bis-ANS, which degrades over long timeframes as Aβ fibrils form.[47] We have shown that this intensity increase can be reversed by the addition of chelators, such as ethylenediaminetetraacetic acid (EDTA), suggesting the increase is due to the formation of a transient species stabilized

by the presence of a metal ion (B. Roberts, Z. Datki and A. I. Bush, unpublished data). In the present study, we incubated Aβ in the presence of bis-ANS and compound, and then added zinc(II) to initiate the formation of bis-ANS positive Aβ oligomers. We conducted multiple controls to test for bis-ANS reactivity in the compounds alone and corrected all traces for any baseline we observed in these samples. We again see a diversity of effects of the added compounds on fibril formation ranging from (1) little effect (compound **26**), (2) partial inhibition (compounds **6**, **19**, **20**, **22**–**24** and **27**–**29**), and (3) complete inhibition (compounds **1**–**5**, **21** and **25**) (Figure 5). In no case did we observe enhancement of the bis-ANS fluorescence (Figure S6), suggesting that these compounds did not enhance the formation of zinc(II)-induced partially folded and misfolded Aβ oligomers. The fact that compounds **1**–**6** inhibited the zinc(II) induced increase in bis-ANS fluorescence is unsurprising as these compounds are chelators and may thus act to compete with Aβ for the zinc(II), thereby removing the driving force for the formation of oligomeric species in the assay. However, the metallated complexes **19**–**25**, **28** and **29** should not have a chelating activity and thus a different mechanism must be invoked to explain the inhibition of the fluorescence intensity increase. One potential explanation is that the compounds interact with Aβ through a fairly specific binding interaction and either displace the bound zinc(II) or suppress the formation of bis-ANS positive oligomeric species of Aβ. As indicated for the tyrosine fluorescence assay, these results support this hypothesis and are interesting, but more work needs to be conducted to validate the presence of such an interaction and determine the consequent effects.

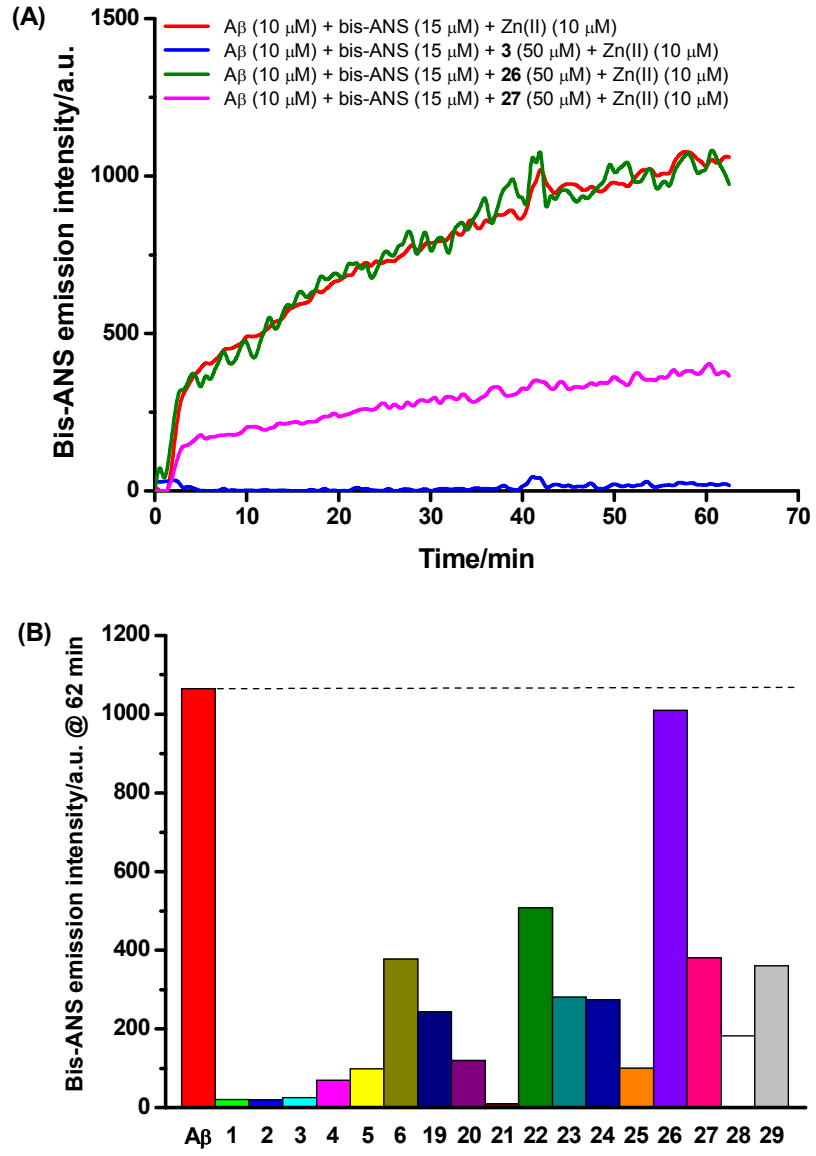


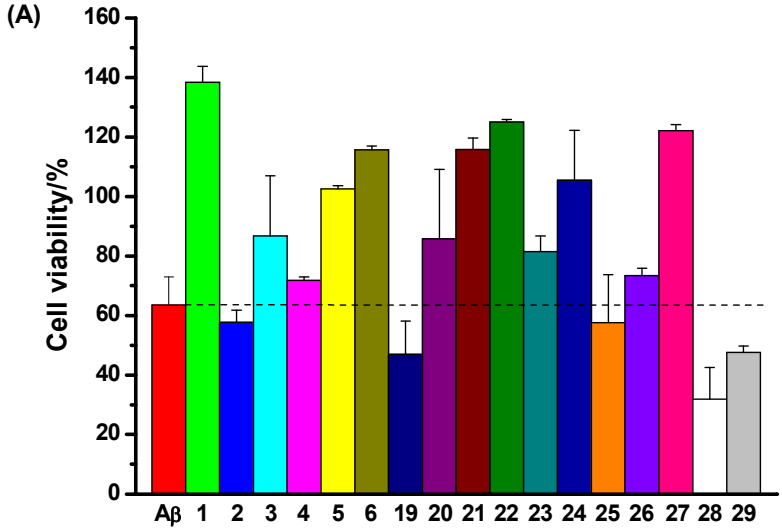
Figure 5. (A) Selected data from bis-ANS extrinsic fluorescence assay: incubation of compound **3**, **26**, or **27** (50 μM) with Aβ (10 μM), bis-ANS (15 μM) and zinc(II) (10 μM) completely quenched (blue), exerted little influence on (green), or reduced (pink) the bis-ANS fluorescence respectively with comparison to that obtained from an incubated solution of Aβ (10 μM), bis-ANS (15 μM) and zinc(II) (10 μM) alone (red); (B) Summary of the effects of compounds **1**–**6** and **19**–**29** on the bis-ANS fluorescence intensity at the end of the assay.

(v) Neurotoxicity Assay

Given the reported prevention of Aβ-mediated toxicity in neuronal cell culture by a cyclen-KLVFF hybrid,[19] a neurotoxicity assay was conducted to examine whether compounds **1**–**6** and **19**–**29** could protect neurons from Aβ toxicity. First SH-SY5Y neuronal cells were incubated in the absence and presence of Aβ (0, 1, 5 and 10 μM) for

4 days. Neuronal viability was assessed by a Resazurin-based fluorescence assay, revealing that Aβ exhibited neurotoxicity only at 10 μM (Figure S7, Supporting Information). Accordingly, this concentration was used in subsequent experiments.

SH-SY5Y neuronal cells were incubated with Aβ (10 μM) and the test compounds (50 μM) for 4 days; neuronal viability was then assessed using the same fluorescence assay (Figure 6A). It was found that (1) most of these compounds (**1**, **3**, **5**, **6**, **20**–**24** and **27**) rescued SH-SY5Y neuronal cells from Aβ-induced neurotoxicity; (2) three compounds (**2**, **4** and **26**) exerted little protective effect on neurons; (3) four compounds marginally (**19**, **25**) or clearly (**28** and **29**) exacerbated the total neurotoxicity. Incubation of SH-SY5Y neuronal cells with the test compounds (50 μM) in the absence of Aβ suggested that only compounds **19**, **25** and **28** themselves were cytotoxic (Figure 6B), which correlates well with the heightened neurotoxicity observed with these compounds in the Aβ neurotoxicity assay.



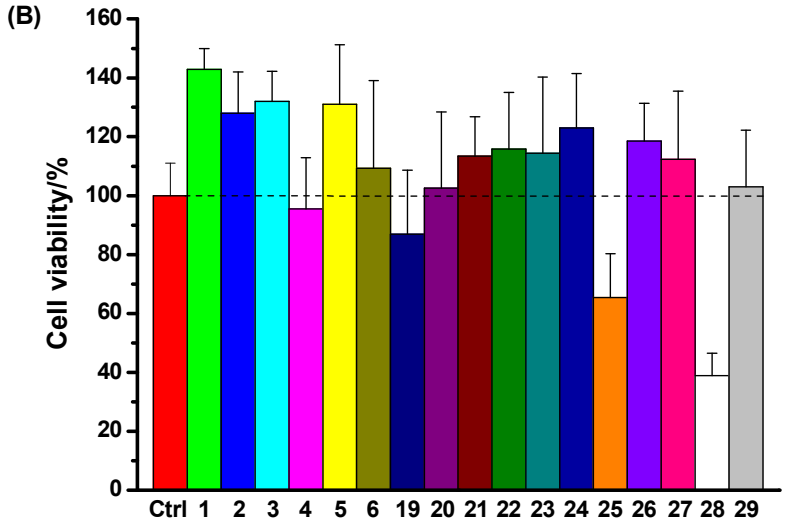


Figure 6. (A) Effects of compounds **1**–**6** and **19**–**29** on Aβ-mediated toxicity in neuronal cell culture; (B) Cytotoxicity of compounds **1**–**6** and **19**–**29** in neuronal cell culture in the absence of Aβ (Ctrl = SH-SY5Y neuron cells only (no test compound was added)).

Experimental

Synthesis and Characterization of Tetraazamcrocycle-(G)KLVFF Hybrids and their Metal Complexes

See Electronic Supplementary Information (ESI) for complete experimental procedures and spectroscopic data.

$A\beta_{42}$ Stock Solution Preparation

$A\beta_{42}$ (a lyophilized powder, the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University) (1 mg) was pre-treated with ammonium hydroxide as described previously.[30] The treated peptides were suspended in 60 mM NaOH (200 µL) and incubated for 5 min at room temperature. The resulting solution was diluted with Milli-Q water (700 µL) and sonicated at room temperature for a further 5 min in a water bath. The sonicated solution was neutralized with 10 × PBS (PBS is defined as 50 mM sodium phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4, 100 µL) and centrifuged for 10 min at 14000 × *g* in a benchtop centrifuge. The optical density at 214 nm of the supernatant, containing the resolubilized $A\beta_{42}$, was determined using a quartz

microplate and a Flexstation 3 microplate reader (Molecular Devices) equipped with absorbance optics. The concentration was calculated from the 214 nm absorbance value using the molar extinction coefficient for $A\beta_{42}$ of 94530 M^{-1} cm^{-1}.[34] Recovery of the peptide was typically 70-80%. The $A\beta_{42}$ stock solution was immediately used for ThT extrinsic fluorescence assay, MALDI-TOF-MS, tyrosine intrinsic fluorescence assay, bis-ANS extrinsic fluorescence assay and neurotoxicity assay.

ThT Extrinsic Fluorescence Assay

The effects of compounds **1**-**6** and **19**-**29** on the $A\beta_{42}$ aggregation was evaluated using a continuous ThT fluorescence assay described previously.[33,34] Compounds were dissolved in DMSO (Sigma Aldrich) to yield 1 mM stocks. These solutions were added into a 96-well microtitre plate (Wallac) containing ThT (Sigma Aldrich) and PBS (pH 7.4) to give final compound concentrations of 10 and 50 μM with a ThT level of 20 μM; $A\beta_{42}$ was added to give a final concentration of 5 μM. The plate was sealed with acetate adhesive seals (MP Biomedicals) to minimize evaporative loss, and incubated at 37 °C for 23 h. During incubation, the plate was shaken every 7 min for 3 s prior to the measurement of the ThT fluorescence intensity (excitation at 444 nm and emission at 485 nm) using a Flexstation 3 microplate reader (Molecular Devices).

$A\beta_{42}$ pelleting assay

The results of the ThT assay were confirmed using a pelleting assay. Samples were prepared and treated as described for the ThT assay, and incubated at 37 °C for 24 h with shaking every 7 min for 3 s using an orbital plate shaker (350 opm). 100 μL of the sample was centrifuged at 100,000 × *g* (Beckman Coulter, TL-100 benchtop ultracentrifuge), and the supernatant, pellet and starting material were assayed for protein concentration using a microBCA assay (Pierce) according to the manufacturer's instructions. The results were confirmed using the Direct Detect protein quantitation instrument from Millipore.

MALDI-TOF-MS

The proteolytic effects of compounds **1**–**6** and **19**–**29** on $A\beta_{42}$ were investigated using MALDI-TOF-MS as described previously.[19] The test compound (50 μM) was incubated

with or without $A\beta_{42}$ (10 μM) in PBS buffer (pH 7.4) for 4 and 7 days at 37 °C under sterile conditions. Each sample was desalted by a reverse phase C18 Zip-tip, and the resulting solution (1 μL) was mixed 1:1 with matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in a mixture of acetonitrile/water/TFA (50:49.9:0.1)) and spotted onto a ground steel target (MTP 384, Bruker Daltonics). Spotted samples were analyzed using a Bruker Daltonics Ultraflex III MALDI-TOF in reflector mode, with a detection range of 900-5000 Da, using appropriate peptide calibrants (Bruker Daltonics). Collected data were baseline corrected and smoothed using the Flexanalysis software module (Bruker Daltonics).

Tyrosine Intrinsic Fluorescence Assay

Tyrosine intrinsic fluorescence assay[19,40,42] was employed to investigate the effects of compounds **1**-**6** and **19**-**29** on the interaction between $A\beta_{42}$ and copper(II). The tyrosine fluorescence spectra (excitation at 275 nm and emission at 290-305 nm) of $A\beta_{42}$ (10 μM) in Tris buffer (50 mM, pH 7.5) in a 96-well microtitre plate (Wallac) were acquired using a Flexstation 3 microplate reader (Molecular Devices) immediately after dissolution. After incubation for 1 h at 25 °C, fluorescence was measured again. Copper(II) chloride was added to give a final concentration of 16 μM, and the fluorescence spectra of the resulting mixtures were acquired immediately. After incubation for 1 h at 25 °C, fluorescence was measured again. The test compounds (1 mM stocks in DMSO) were added to give a final concentration of 40 μM, and the fluorescence spectra of the resulting mixtures were acquired immediately. After incubation for 1 h at 25 °C, fluorescence was measured again.

Bis-ANS Extrinsic Fluorescence Assay

The ability of compounds **1**–**6** and **19**–**29** to interfere with zinc(II) related enhancement of Aβ self-association was analyzed using the dye 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS). The assay was conducted by adding bis-ANS, $A\beta_{42}$ and compound to a microtiter plate containing 1 × PBS (200 μL) to final concentrations of 15, 10 and 50 μM respectively at 25 °C. Upon mixing, the fluorescence emission was recorded at 485 nm with excitation at 390 nm using a Flexstation 3 Plate reader (Molecular Devices, Sunnyvale, California) at 1 minute intervals. After 5 minutes, zinc(II)

was added to a final concentration of 10 μM, and the fluorescence intensity was monitored for a further hour. All data were corrected for the intensity of changes of samples containing no $A\beta_{42}$. Data reduction was done by taking the final point of the 60 minute time course.

Neurotoxicity Assay

Neuronal cell line SH-SY5Y was cultured in RPMI (Invitrogen) supplemented with 10% heat inactivated FBS (Invitrogen), HEPES (25 mM; GIBCO), L-glutamine (100 mM; GIBCO) and β-mercaptoethanol. Cells were seeded at 1×10^5 cells per well of a 96-well microtitre plate (Falcon, BD Biosciences) in 100 μL of culture media and allowed to adhere overnight under standard cell culture conditions (37 °C, 5% CO_2 and 95% humidity). Compounds **1**–**6** and **19**–**29** at a final concentration of 50 μM in 0.1% DMSO (Sigma-Aldrich) were combined with $A\beta_{42}$ at final concentrations of 1, 5 and 10 μM in triplicate in appropriate wells. Cells, media and $A\beta_{42}$ alone were used as controls. The plates were incubated for 96 h (37 °C, 5% CO_2 and 95% humidity). Resazurin (Sigma-Aldrich) was added at a final concentration of 0.05% (w/v) per well and incubated for 4 h before a measurement of fluorescent intensity was recorded on a FLUOstar OMEGA at 540/595 nm. Addition of compounds incorporating zinc(II) or copper(II) directly to resazurin did not alter the output fluorescence after 4 days incubation at 37 °C in 5% CO_2. The percentage cell viability was calculated in relation to the maximum and minimum measurement of fluorescence caused by cells only (100% survival) or media alone (100% inhibition). All experiments were performed in duplicate, n = 2.

Conclusions

Tetraazamacrocycle-(G)KLVFF hybrids **1**–**6** were prepared efficiently in good overall yields using a solid-phase based synthetic procedure. Complexation of these six conjugates with zinc(II) and copper(II) salts proceeded smoothly to give the corresponding metal complexes **19** – **26** in good to excellent yields. The biological activities of these tetraazamacrocycle-(G)KLVFF hybrids and their metal complexes were evaluated *in vitro* using several assays. Both the extrinsic fluorescence assays using ThT and bis-ANS, and the tyrosine intrinsic fluorescence assay imply complex,

multifaceted structure-activity relationships in the interaction of these conjugates and Aβ. None of the compounds tested degrade Aβ into fragments. However most of these compounds protect neurons from Aβ toxicity.

The previously reported ability of compound **1** to capture copper(II) ions and become proteolytically active could not be replicated in the present study. The differences might be explained by the different sources of Aβ or their slightly different methods of preparation. In the previous report, the Aβ was prepared in HFIP, but this was not used here as it has been found that HFIP can increase Aβ aggregation on its own.[30] While it is known that Aβ prepared with alternative pretreatments to HFIP, such as ammonium hydroxide (NH_4OH), differs in its aggregation speed, NH_4OH pretreatment was used in the present work since our previous work shows that this treatment results in an almost monomeric starting solution.[30] The method of preparation of the metal complexes also differed: in the present work the metal complexes were isolated and purified, whereas in the previous work the complexes were prepared *in situ* with a slight excess of the ligand over the metal (1.2:1). The association constants for ligands/metal ions of this general class are high,[48-51] meaning one would expect a vanishingly small free metal ion concentration from a 1:1 mixture of metal and ligand, but the slight excess of the free ligand used in the previous work could mean it is possible there was some free ligand present in the sample when it was biologically evaluated.

More generally for the overall set of compounds described in this paper, the results do not clearly articulate any consistent SARs and instead the data – particularly for the pre-formed metal complexes – suggest that interactions between compounds of this class and Aβ are complex. This is not unexpected given the *a priori* complexity of any binding interaction and what has been learned from the recent use of simple non-metallated peptides in the inhibition of amyloid formation.[52-58] The results from the tyrosine intrinsic fluorescence assay in particular should caution that the changes in optical output observed using this assay may be caused by more complex changes than simple metal sequestration (which certainly should be more likely with the free ligands tested than the pre-formed metal complexes, in contrast the outcomes discussed above). Further work is needed to verify whether other peptide- or chelator-Aβ interactions can give rise to the observed fluorescence quenching. Despite the lack of clear patterns and

SARs from the fluorescence and proteolytic assays with isolated protein, many of the compounds screened demonstrated an impressive ability to rescue cells from Aβ-induced cytotoxicity. The attendant simplicity and ready diversification of their synthesis makes this a promising class of compounds for further investigation.

Acknowledgements

We thank the National Breast Cancer Fund for a Novel Concept Grant (NC-10-69) and the Australian Research Council for funding (DP120104035). M. Yu was supported by a University of Sydney International Scholarship (USydIS). T. M. Ryan is on a fellowship with the Australia Alzheimer's Dementia Research Foundation (AADRF).

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Electronic Supplementary Information (ESI)

1. General Materials

All reactions except solid phase peptide synthesis were carried out with continuous magnetic stirring in ordinary glassware; solid phase peptide synthesis was performed in 10 mL polypropylene syringes with filters, purchased from Torviq, on an IKA® VXR basic Vibrax® shaker. Heating of reactions was conducted with a paraffin oil bath; cooling of reactions was achieved using an ice or ice-salt bath. All reagents and solvents were purchased from Sigma-Aldrich, Alfa Acer, Merck, Mimotopes, GL Biochem or Ajax Finechem. Wang resin was purchased from Novabiochem. Reagents were used as received unless otherwise specified. Hexane and ethyl acetate were distilled before use. Dichloromethane and ethanol were distilled over calcium hydride and stored over activated 4 Å molecular sieves. Chloroform was passed through a column of basic alumina prior to use. Diethyl ether, methanol, acetonitrile and *N*,*N*-dimethylformamide were collected freshly from a PureSolv MD 7 solvent purification system having been passed through anhydrous alumina columns.

2. Instrumentation and Methods

^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker AVANCE 200 spectrometer (^{1}H at 200.13 MHz and ^{13}C at 50.32 MHz), a Bruker AVANCE 300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.47 MHz) or a Bruker DRX 400 spectrometer (^{1}H at 400.13 MHz and ^{13}C at 100.61 MHz). ^{1}H and ^{13}C NMR spectra are referenced to ^{1}H signals of residual nondeuterated solvents (or tetramethylsilane) and ^{13}C signals of the deuterated solvents respectively. ^{1}H NMR signals are reported with chemical shift values δ (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, m = multiplet and br = broad), relative integral, coupling constants J (Hz) and assignments. Infrared spectra were recorded on a Bruker Alpha FT-IR spectrometer. UV-Vis spectra were recorded on a Varian Cary 4000 or Varian Cary 1E UV-visible spectrophotometer. Temperature control for UV-visible spectrophotometer was provided by a Varian Cary PCB water peltier system. Low resolution and high resolution mass spectra were recorded on a Finnigan LCQ mass spectrometer and a Bruker 7T Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass Spectrometer respectively. Ionisation of all samples was carried out using ESI. Optical rotation α was measured on

a PerkinElmer 341 polarimeter with a sodium lamp in a semi-micro fused silica polarimeter cell (length: 100 mm, capacity: 3.0 mL) at 589 nm and 20 °C using spectroscopic grade solvents. Temperature was controlled by a Julabo F12-ED refrigerated/heating circulator connected directly to the polarimeter cell. Melting points were determined on an OptiMelt 100 automated melting point apparatus and are uncorrected. Elemental analyses were carried out by the Campbell Microanalytical Laboratory (University of Otago, New Zealand) on a Carlo Erba EA 1108 Elemental Analyser. Analytic reverse phase high performance liquid chromatography (RP-HPLC) was carried out on a Waters 2695 separations module with a Waters 2996 photodiode array detector and an Alliance series column heater. A Waters SunFire™ C18 column (5 µm, 2.1 × 150 mm) was used at 30 °C at a flow rate of 0.2 mL/min. Preparative RP-HPLC was carried out on a Waters 600 controller with a Waters 600 pump and a 2998 photodiode array detector. A Waters SunFire™ C18 OBD™ column (5 µm, 19 × 150 mm) was used at a flow rate of 7 mL/min. Mobile phases of 0.1% TFA in Milli-Q water (solvent A) and 0.1% TFA in acetonitrile (solvent B) in different ratios was used in both analytic and preparative HPLC. The fractions from preparative HPLC were lyophilized using a Labconco FreeZone 6 liter console freeze dry system. Data acquired from both analytic and preparative HPLC were processed using Waters Empower 2 software. Liquid chromatography mass spectrometry (LCMS) was performed on a Thermo Separation Products: Spectra System consisting of a P400 pump and a UV6000LP photodiode array detector coupled to a Thermoquest Finnigan LCQ Deca mass spectrometer (ESI). A Phenomenex Jupiter C18 column (5 µm, 2.1 × 150 mm) was eluted at a flow rate of 0.2 mL/min with a mobile phase of 0.1% formic acid in Milli-Q water and 0.1% formic acid in acetonitrile. Analytical TLC was performed on Merck silica gel 60 F_{254} pre-coated aluminium plates (0.2 mm) and visualized under UV light (254 nm), followed by staining with ninhydrin. Flash column chromatography was carried out using Merck silica gel 60 (0.040-0.063 mm).

3. General Synthetic Procedures

General Synthetic Procedure A: SPPS of Peptides following the Fmoc Strategy[59,60]

Pre-loading of Wang Resin

Wang resin (1.0 eq.) was washed with DMF (5 ×), DCM (5 ×) and DMF (5 ×), and swelled in DMF for 30 min before use. Fmoc-Phe-OH (10.0 eq.) was dissolved in anhydrous DCM (0.1 M) and cooled to 0 °C. DIC (5.0 eq.) was added dropwise. The reaction mixture was stirred for 30 min at 0 °C and concentrated under reduced pressure. The residue and DMAP (0.1 eq.) were dissolved in DMF (final concentration 0.1 M) and added immediately to the pre-swelled Wang resin. The resin was shaken for 2 h and washed with DMF (5 ×), DCM (5 ×) and DMF (5 ×). Capping with acetic anhydride/pyridine (1:9, v/v) (2 × 5 min) was followed by washing with DMF (5 ×), DCM (5 ×) and DMF (5 ×). Treatment of the resin with 10% piperidine/DMF (2 × 5 min) and measurement of the absorbance of the resulting piperidine-fulvene adduct at λ = 301 nm showed that the resin loading was quantitative.

Iterative Peptide Assembly

Deprotection: The resin was treated with 10% piperidine/DMF (2 × 5 min) and washed with DMF (5 ×), DCM (5 ×) and DMF (5 ×).

Amino acid coupling: A pre-activated solution of Fmoc-protected amino acid (4.0 eq.), PyBOP (4.0 eq.) and NMM (8.0 eq.) in DMF (final concentration 0.1 M) was added to the resin. After shaking for 1 h, the resin was washed with DMF (5 ×), DCM (5 ×) and DMF (5 ×).

Capping: The resin was treated with acetic anhydride/pyridine (1:9, v/v) (2 × 5 min) and washed with DMF (5 ×), DCM (5 ×) and DMF (5 ×).

Acetic acid derivative coupling: A pre-activated solution of an acetic acid derivative (**9**, **10**, **11** or **12**) (4.0 eq.), PyBOP (4.0 eq.) and NMM (8.0 eq.) in DMF (final concentration 0.1 M) was added to the resin. After shaking for 1 h, the resin was washed with DMF (5 ×) and DCM (10 ×) and dried *in vacuo*. The capping and deprotection steps were omitted.

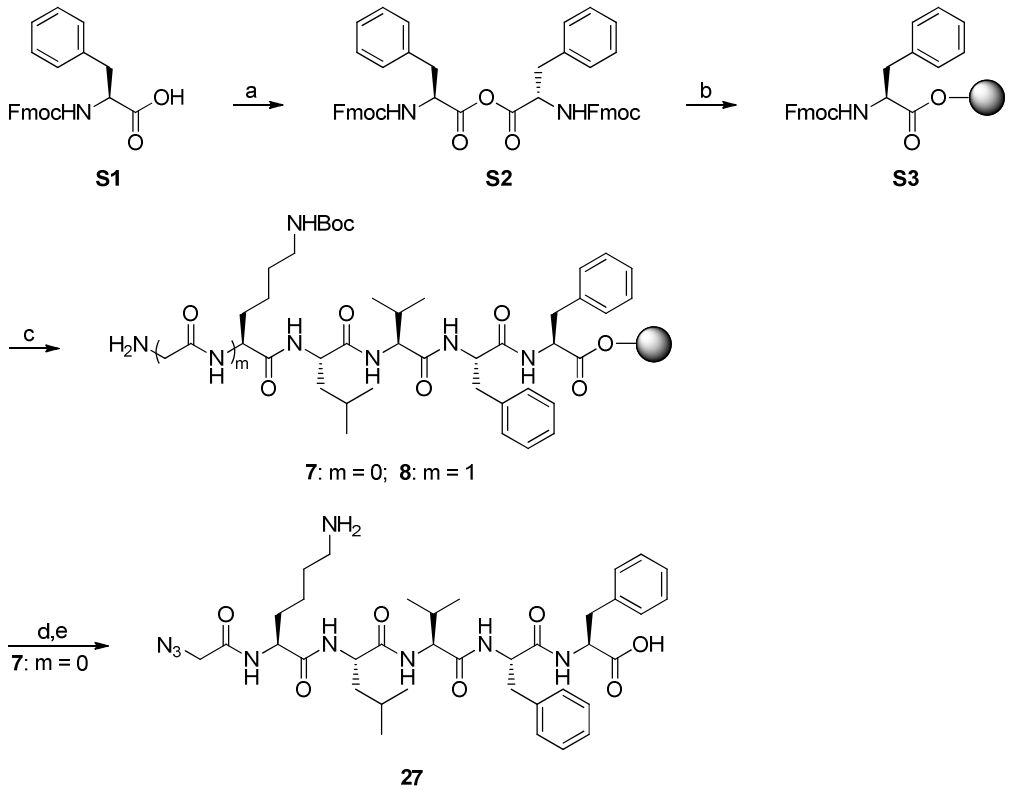
Cleavage: A mixture of TFA/TIS/H_2O (90:5:5, v/v/v) was added to the resin. After shaking for 2 h, the resin was washed with TFA (3 × 5 mL).

Work-up: The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC.

General Synthetic Procedure B: Metal Complexation[25]

To a solution of *N*-functionalized cyclam trifluoroacetate (1.0 eq.) in EtOH (0.1 M) was added dropwise a solution of $CuCl_2 \cdot 2H_2O$ or $ZnCl_2$ (1.0 eq.) in EtOH (0.1 M) at room temperature. The reaction mixture was heated at reflux for 6 h and cooled on an ice bath. The desired metal complex was isolated from the suspension by centrifugation.

4. Synthesis of Precursors 7-12 and the Control Compound 27

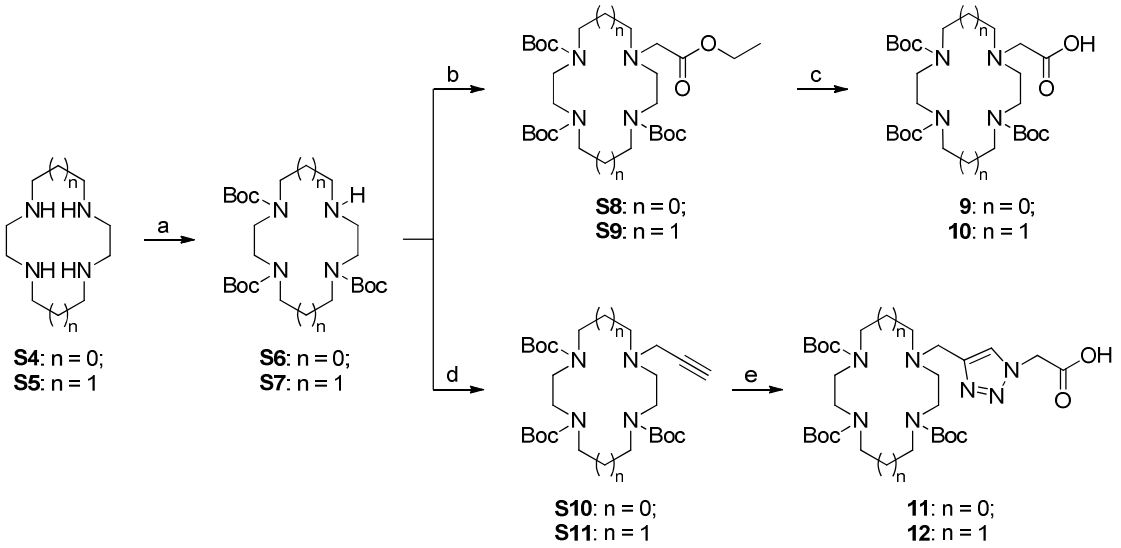


Scheme S1. Synthesis of resin-bound oligopeptides **7** and **8** as well as the control compound **27**. Reagents and conditions: (a) DIC, DCM, 0 °C, 1 h; (b) Wang resin, DMAP, DMF, rt, 2 h; (c) iterative Fmoc strategy SPPS (4 times for **7** and 5 times for **8**): (1) Fmoc removal: 10% piperidine/DMF, rt, 2 × 5 min; (2) amino

acid coupling: Fmoc-X_{aa}-OH (X_{aa} = Phe, Val, Leu, Lys(Boc) and Gly), PyBOP, NMM, DMF, rt, 1 h; (3) capping: 10% Ac_2O/pyridine, rt, 2 × 5 min; (d) only for **7**, 2-azidoacetic acid, PyBOP, NMM, DMF, rt, 1 h; (e) TFA/TIS/H_2O (90:5:5), rt, 2 h, followed by RP-HPLC purification, 72%.

(2*S*,5*S*,8*S*,11*S*,14*S*)-14-(4-Aminobutyl)-17-azido-2,5-dibenzyl-11-isobutyl-8-isopropyl-4,7,10,13,16-pentaoxo-3,6,9,12,15-pentaazaheptadecan-1-oic acid (27).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 182 mg, 0.200 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and azide-capped pentapeptide **27** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 10% to 50% B over 45 min) to give **27** as a white solid (106 mg, 72%). **m.p.** 238-239 °C. $\mathbf{[\alpha]_D^{20}}$ -22.5 (*c* 1.0, DMSO). **IR** ν_{max}/cm^{-1} 3277, 3074, 3028, 2956, 2875, 2108, 1630, 1540, 1429, 1399, 1281, 1198, 1137, 1036, 694. **^{1}H NMR** (500 MHz, CD_3OD) δ 0.78 (d, 3H, *J* 7.0, CH_3), 0.83 (d, 3H, *J* 6.5, CH_3), 0.88 (d, 3H, *J* 6.0, CH_3), 0.93 (d, 3H, *J* 6.5, CH_3), 1.39-1.46 (m, 2H), 1.46-1.51 (m, 1H), 1.55-1.60 (m, 1H), 1.60-1.72 (m, 4H), 1.78-1.86 (m, 1H), 1.91-1.99 (m, 1H) (total 10H, CHC*H*$(CH_3)_2$ & CH_2C*H*$(CH_3)_2$ & CH_2CH_2CH_2CH_2NH_2), 2.85 (dd, 1H, *J* 14.0 & 9.5, C*H*HPh), 2.89 (t, 2H, *J* 7.5, CH_2NH_2), 3.00 (dd, 1H, *J* 14.0 & 8.0, CH*H*Ph), 3.09 (dd, 1H, *J* 14.0 & 5.5, C*H*HPh), 3.17 (dd, 1H, *J* 14.0 & 5.5, CH*H*Ph), 3.90 (s, 2H, N_3CH_2), 4.16 (t, 1H, *J* 8.0, NHC*H*CO), 4.41-4.50 (m, 2H, 2 × NHC*H*CO), 4.62-4.71 (m, 2H, 2 × NHC*H*CO), 7.15-7.45 (m, 10H, Ph-H), 7.99 (d, 1H, *J* 8.5, CONH), 8.11 (d, 1H, *J* 8.0, CONH), 8.20 (d, 1H, *J* 7.5, CONH), 8.28 (d, 1H, *J* 7.5, CONH) (two primary amine proton signals (NH_2), one amide proton signal (CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, CD_3OD) δ 18.8, 19.8, 22.0, 23.5, 23.6, 25.8, 28.1, 32.3, 32.7, 38.5, 39.1, 40.5, 41.7, 52.7, 53.4, 54.3, 55.1, 55.6, 60.1, 127.7, 127.8, 129.4, 129.5, 130.3, 138.2, 170.2, 172.9, 173.0, 173.6, 174.2, 174.5 (six carbon signals overlapping or obscured). **MS** (ESI) *m/z* 736.1 ([M+H]$^+$, 100%), 758.2 ([M+Na]$^+$, 6%), 1471.1 ([2M+H]$^+$, 19%). **HRMS** (ESI) 736.41304 ([M+H]$^+$); calcd. for $C_{37}H_{54}N_9O_7$ ([M+H]$^+$) 736.41407. **Anal.** Calcd. for $C_{37}H_{53}N_9O_7 \cdot CF_3COOH \cdot H_2O$: C 53.97, H 6.50, N 14.52; Found: C 54.06, H 6.51, N 14.49.



Scheme S2. Synthesis of precursors **9**-**12**. Reagents and conditions: (a) Boc_2O, Et_3N, $CHCl_3$ for **S4** and DCM for **S5**, 0 °C to rt, o/n, **S6**: 72%, **S7**: 77%; (b) $BrCH_2COOCH_2CH_3$, Na_2CO_3, CH_3CN, reflux, o/n, **S8**: 100%, **S9**: 91%; (c) 1 M NaOH, CH_3OH, rt, 2 h for **9** and 2.5 h for **10**, **9**: 100%, **10**: 93%; (d) propargyl bromide, Na_2CO_3, CH_3CN, reflux, o/n, **S10**: 96%, **S11**: 95%; (e) 2-azidoacetic acid, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, *t*-BuOH/H_2O (1:1), rt, o/n, **11**: 100%, **12**: 98%.

Tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (S6).[61-63]

To a solution of cyclen (**S4**, 1.73 g, 10.0 mmol) and triethylamine (4.20 mL, 30.1 mmol) in $CHCl_3$ (120 mL, freshly passed through Al_2O_3 (activated, neutral, Brockmann I)) at 0 °C was added dropwise a solution of di-*tert*-butyl dicarbonate (6.55 g, 30.0 mmol) in $CHCl_3$ (100 mL, freshly passed through Al_2O_3 (activated, neutral, Brockmann I)) under N_2. After the addition was complete, the resulting solution was allowed to warm to room temperature and stirred overnight. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 3:2 ramping to EtOAc) to give **S6** as a white foam (3.41 g, 72%). $\boldsymbol{R_F}$ (EtOAc:hexane = 4:1) 0.63. **IR** ν_{max}/cm^{-1} 3313, 2974, 2931, 2818, 1679, 1463, 1412, 1365, 1313, 1247, 1156, 1046, 771, 736. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.45 (s, 18H, 2 × $C(CH_3)_3$), 1.47 (s, 9H, $C(CH_3)_3$), 2.78-2.92 (m, 4H, CH_2NHCH_2), 3.16-3.34 (m, 6H), 3.34-3.50 (m, 2H), 3.55-3.75 (m, 4H) (total 12H, 3 × $CH_2N(Boc)CH_2$) (one secondary amine proton signal (NH) not observed). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 28.1, 28.2, 28.3, 28.4, 28.5, 44.7, 45.7, 48.8, 49.2, 50.3, 50.8, 78.9, 79.1, 155.1, 155.4 (eight carbon signals overlapping or obscured). **MS** (ESI) *m/z* 472.9 ($[M+H]^+$, 27%), 495.0 ($[M+Na]^+$, 99%),

967.1 ([2M+Na]$^+$, 100%). The spectroscopic data were in agreement with those in the literature.[61-63]

Tri-*tert*-butyl 1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate (S7).[64]

To a solution of cyclam (**S5**, 1.51 g, 7.54 mmol) and triethylamine (5.20 mL, 37.3 mmol) in anhydrous DCM (300 mL) was added dropwise di-*tert*-butyl dicarbonate (2.95 g, 13.5 mmol) in anhydrous DCM (90 mL) under N_2. After the addition was complete, the reaction mixture was cooled to -15 °C, and a second portion of di-*tert*-butyl dicarbonate (1.96 g, 8.98 mmol) in anhydrous DCM (60 mL) was added. The reaction mixture was stirred at room temperature overnight and washed with 0.5 M Na_2CO_3 (2 × 150 mL). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc ramping to EtOAc:CH_3OH = 9:1) to give **S7** as a white foam (2.91 g, 77%). $\boldsymbol{R_F}$ (EtOAc:CH_3OH = 9:1) 0.54. **m.p.** 46-47 °C. **IR** ν_{max}/cm^{-1} 2973, 2932, 2818, 1681, 1464, 1409, 1389, 1364, 1239, 1158. **^{1}H NMR** (200 MHz, $CDCl_3$) δ 1.46 (s, 27H, 3 × $C(CH_3)_3$), 1.60-1.80 (m, 2H, $CH_2CH_2CH_2$), 1.80-2.10 (m, 2H, $CH_2CH_2CH_2$), 2.62 (t, 2H, *J* 5.6, CH_2NHCH_2), 2.78 (t, 2H, *J* 5.4, CH_2NHCH_2), 3.20-3.50 (m, 12H, 3 × $CH_2N(Boc)CH_2$) (one secondary amine proton signal (NH) not observed). **MS** (ESI) *m/z* 501.3 ([M+H]$^+$, 100%), 523.5 ([M+Na]$^+$, 17%). The spectroscopic data were in agreement with those in the literature.[64]

Tri-*tert*-butyl 10-(2-ethoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (S8).[65]

To a solution of tri-Boc cyclen **S6** (6.04 g, 12.8 mmol) in anhydrous CH_3CN (120 mL) were added Na_2CO_3 (1.63 g, 15.4 mmol) and ethyl bromoacetate (1.70 mL, 15.3 mmol). The reaction mixture was stirred at reflux under N_2 overnight. The insoluble salts were filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 1:2 ramping to 1:1) to give **S8** as a white foam (7.14 g, 100%). $\boldsymbol{R_F}$ (EtOAc:hexane = 1:1) 0.71. **IR** ν_{max}/cm^{-1} 2975, 2932, 1735, 1682, 1459, 1413, 1364, 1312, 1248, 1156, 1030, 770. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.27 (t, 3H, *J* 6.8, $COOCH_2CH_3$), 1.45 (s, 18H, 2 × $C(CH_3)_3$), 1.48 (s, 9H, $C(CH_3)_3$), 2.85-3.02 (m, 4H, $CH_2N(CH_2COOCH_2CH_3)CH_2$), 3.20-3.65 (br m, 12H, 3 ×

$CH_2N(Boc)CH_2$), 3.51 (s, 2H, $NCH_2COOCH_2CH_3$), 4.15 (q, 2H, *J* 6.8, $COOCH_2CH_3$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 13.9, 28.1, 28.3, 46.7, 47.0, 47.3, 49.5, 50.7, 53.2, 54.5, 59.8, 78.7, 79.0, 79.1, 154.9, 155.3, 155.6, 170.1 (nine carbon signals overlapping or obscured). **MS** (ESI) *m/z* 581.0 ([M+Na]$^+$, 100%), 1139.0 ([2M+Na]$^+$, 98%). The spectroscopic data were in agreement with those in the literature.[65]

Tri-*tert*-butyl 11-(2-ethoxy-2-oxoethyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate (S9).[66,67]

To a solution of tri-Boc cyclam **S7** (3.80 g, 7.59 mmol) in anhydrous CH_3CN (160 mL) were added Na_2CO_3 (0.956 g, 9.10 mmol) and ethyl bromoacetate (1.00 mL, 9.02 mmol). The reaction mixture was stirred at reflux under Ar overnight. The insoluble salts were filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 1:2 ramping to 1:1) to give **S9** as a white foam (4.06 g, 91%). R_F (EtOAc:hexane = 1:1) 0.67. **IR** ν_{max}/cm^{-1} 2974, 2933, 2869, 1737, 1685, 1465, 1411, 1366, 1292, 1240, 1154, 1032, 772, 731. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 1.26 (t, 3H, *J* 7.2, $COOCH_2CH_3$), 1.46 (s, 27H, 3 × $C(CH_3)_3$), 1.60-1.78 (m, 2H, $CH_2CH_2CH_2$), 1.85-2.00 (m, 2H, $CH_2CH_2CH_2$), 2.60-2.72 (m, 2H, $CH_2N(CH_2COOCH_2CH_3)CH_2$), 2.80-2.90 (m, 2H, $CH_2N(CH_2COOCH_2CH_3)CH_2$), 3.22-3.65 (m, 14H, 3 × $CH_2N(Boc)CH_2$ & $NCH_2COOCH_2CH_3$), 4.14 (q, 2H, *J* 7.2, $COOCH_2CH_3$). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 14.2, 27.0, 28.4, 45.2, 46.8, 47.1, 47.3, 48.3, 51.8, 52.9, 53.6, 55.3, 60.1, 79.4, 155.4, 155.6, 170.9 (twelve carbon signals overlapping or obscured). **MS** (ESI) *m/z* 587.0 ([M+H]$^+$, 6%), 609.1 ([M+Na]$^+$, 100%), 1194.9 ([2M+Na]$^+$, 47%). The spectroscopic data were in agreement with those in the literature.[66,67]

2-(4,7,10-Tris(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (9).[65]

To a solution of ester **S8** (559 mg, 1.00 mmol) in CH_3OH (10 mL) was added 1 M NaOH (10 mL). The resulting cloudy reaction mixture was stirred at room temperature for 2 h and concentrated under reduced pressure. The residue was dissolved in 10% citric acid, taken to pH 5 and extracted with EtOAc (2 × 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give **9** as a white foam

(531 mg, 100%). The product was of sufficient purity to be used directly in the next step, but an analytical sample could be obtained by flash column chromatography (silica gel, EtOAc:hexane = 1:1 ramping to EtOAc). R_F (EtOAc:CH_3OH = 9:1) 0.54. **m.p.** 98-99 °C (lit.[68] **m.p.** 138 °C). **IR** ν_{max}/cm^{-1} 3505, 2974, 2931, 2869, 1738, 1682, 1462, 1414, 1366, 1250, 1156, 1115, 1038, 976, 856, 770. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.45 (s, 18H, 2 × $C(CH_3)_3$), 1.48 (s, 9H, $C(CH_3)_3$), 2.85-3.05 (m, 4H, $CH_2N(CH_2COOH)CH_2$), 3.25-3.50 (m , 8H), 3.50-3.65 (m, 6H) (total 14 H, 3 × $CH_2N(Boc)CH_2$ & NCH_2COOH), 9.90 (br s, 1H, COOH). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 28.3, 28.5, 47.2, 47.5, 49.7, 51.0, 54.0, 79.4, 79.7, 155.3, 155.9, 172.8 (thirteen carbon signals overlapping or obscured). **MS** (ESI+) *m/z* 531.0 ($[M+H]^+$, 22%), 553.1 ($[M+Na]^+$, 65%), 1083.0 ($[2M+Na]^+$, 100%); (ESI-) *m/z* 529.2 ($[M-H]^-$, 50%), 1059.3 ($[2M-H]^-$, 100%), 1081.7 ($[2(M-H)+Na]^-$, 14%). The spectroscopic data were in agreement with those in the literature.[65,68,69]

2-(4,8,11-Tris(*tert*-butoxycarbonyl)-1,4,8,11-tetraazacyclotetradecan-1-yl)acetic acid (10).[66,67]

To a solution of ester **S9** (3.20 g, 5.45 mmol) in CH_3OH (64 mL) was added 1 M NaOH (40 mL). The reaction mixture was stirred at room temperature for 2.5 h and concentrated under reduced pressure. The residue was dissolved in 10% citric acid, taken to pH 5 and extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc ramping to EtOAc:CH_3OH = 9:1) to give **10** as a white foam (2.83 g, 93%). R_F (EtOAc:CH_3OH = 8:2) 0.17. **m.p.** 65-66 °C (lit.[67] **m.p.** 89-91 °C). **IR** ν_{max}/cm^{-1} 2974, 2932, 1680, 1468, 1413, 1367, 1304, 1242, 1154, 1060, 912, 727. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 1.46 (s, 27H, 3 × $C(CH_3)_3$), 1.75-1.85 (m, 2H, $CH_2CH_2CH_2$), 1.85-2.00 (m, 2H, $CH_2CH_2CH_2$), 2.75-2.85 (m, 2H, $CH_2N(CH_2COOH)CH_2$), 2.90-3.05 (m, 2H, $CH_2N(CH_2COOH)CH_2$), 3.25-3.55 (m, 14H, 3 × $CH_2N(Boc)CH_2$ & NCH_2COOH), 9.06 (br s, 1H, COOH). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 26.5, 28.5, 45.9, 46.5, 47.5, 47.7, 52.5, 53.8, 56.4, 79.8, 80.4, 155.6, 156.3, 172.1 (thirteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 559.0 ($[M+H]^+$, 45%), 581.1 ($[M+Na]^+$, 100%), 1139.2 ($[2M+Na]^+$, 88%). The spectroscopic data were in agreement with those in the literature.[66,67]

Tri-*tert*-butyl 10-(prop-2-yn-1-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (S10).[51]

To a solution of tri-Boc cyclen **S6** (3.17 g, 6.71 mmol) in anhydrous CH_3CN (200 mL) were added Na_2CO_3 (2.85 g, 26.9 mmol) and propargyl bromide (~80% in toluene, 1.20 mL, 8.07 mmol). The reaction mixture was stirred at reflux under N_2 overnight. The insoluble salts were filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 1:1) to give **S10** as a white solid (3.28 g, 96%). R_F (EtOAc:hexane = 1:1) 0.78. **m.p.** 127-128 °C. **IR** ν_{max}/cm^{-1} 3303, 3251, 2974, 2930, 2831, 1677, 1460, 1413, 1365, 1313, 1250, 1157, 1035, 731. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.45 (s, 18H, 2 × $C(CH_3)_3$), 1.47 (s, 9H, $C(CH_3)_3$), 2.21 (s, 1H, C≡CH), 2.65-2.85 (m, 4H, $CH_2N(CH_2C{\equiv}CH)CH_2$), 3.20-3.45 (m, 8H), 3.45-3.65 (m, 4H) (total 12H, 3 × $CH_2N(Boc)CH_2$), 3.53 (s, 2H, $NCH_2C{\equiv}CH$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 28.5, 28.7, 39.0, 46.5, 47.0, 47.7, 47.8, 49.8, 49.9, 53.1, 54.3, 73.7, 77.6, 79.2, 79.4, 79.7, 155.2, 155.7, 156.0 (seven carbon signals overlapping or obscured). **MS** (ESI) *m/z* 533.0 ($[M+Na]^+$, 41%), 1043.1 ($[2M+Na]^+$, 100%). **HRMS** (ESI) 533.33145 ($[M+Na]^+$); calcd. for $C_{26}H_{46}N_4NaO_6$ ($[M+Na]^+$) 533.33096. The spectroscopic data were in agreement with those in the literature.[51]

Tri-*tert*-butyl 11-(prop-2-yn-1-yl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate (S11).[49,70]

To a solution of tri-Boc cyclam **S7** (437 mg, 0.873 mmol) in anhydrous CH_3CN (26 mL) were added Na_2CO_3 (370 mg, 3.49 mmol) and propargyl bromide (~80% in toluene, 156 µL, 1.05 mmol). The reaction mixture was heated at reflux under N_2 overnight. The insoluble salts were filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 7:3) to give **S11** as a white foam (446 mg, 95%). R_F (EtOAc:hexane = 7:3) 0.58. **m.p.** 47-48 °C (lit.[49,70] **m.p.** 47-49 °C). **IR** ν_{max}/cm^{-1} 3305, 3243, 2976, 2932, 2871, 2826, 1681, 1463, 1410, 1365, 1240, 1150. **^{1}H NMR** (200 MHz, $CDCl_3$) δ 1.40 (s, 27H, 3 × $C(CH_3)_3$), 1.55-1.75 (m, 2H, $CH_2CH_2CH_2$), 1.75-1.95 (m, 2H, $CH_2CH_2CH_2$), 2.12 (s, 1H, C≡CH), 2.46 (t, 2H, *J* 5.4, $CH_2N(CH_2C{\equiv}CH)CH_2$), 2.55-2.70 (m, 2H, $CH_2N(CH_2C{\equiv}CH)CH_2$), 3.10-3.50 (br m, 14H, 3 × $CH_2N(Boc)CH_2$ & $NCH_2C{\equiv}CH$). **MS** (ESI) *m/z* 539.4 ($[M+H]^+$, 100%), 561.5

([M+Na]$^+$, 28%). The spectroscopic data were in agreement with those in the literature.[49,70]

2-(4-((4,7,10-Tris(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid (11).

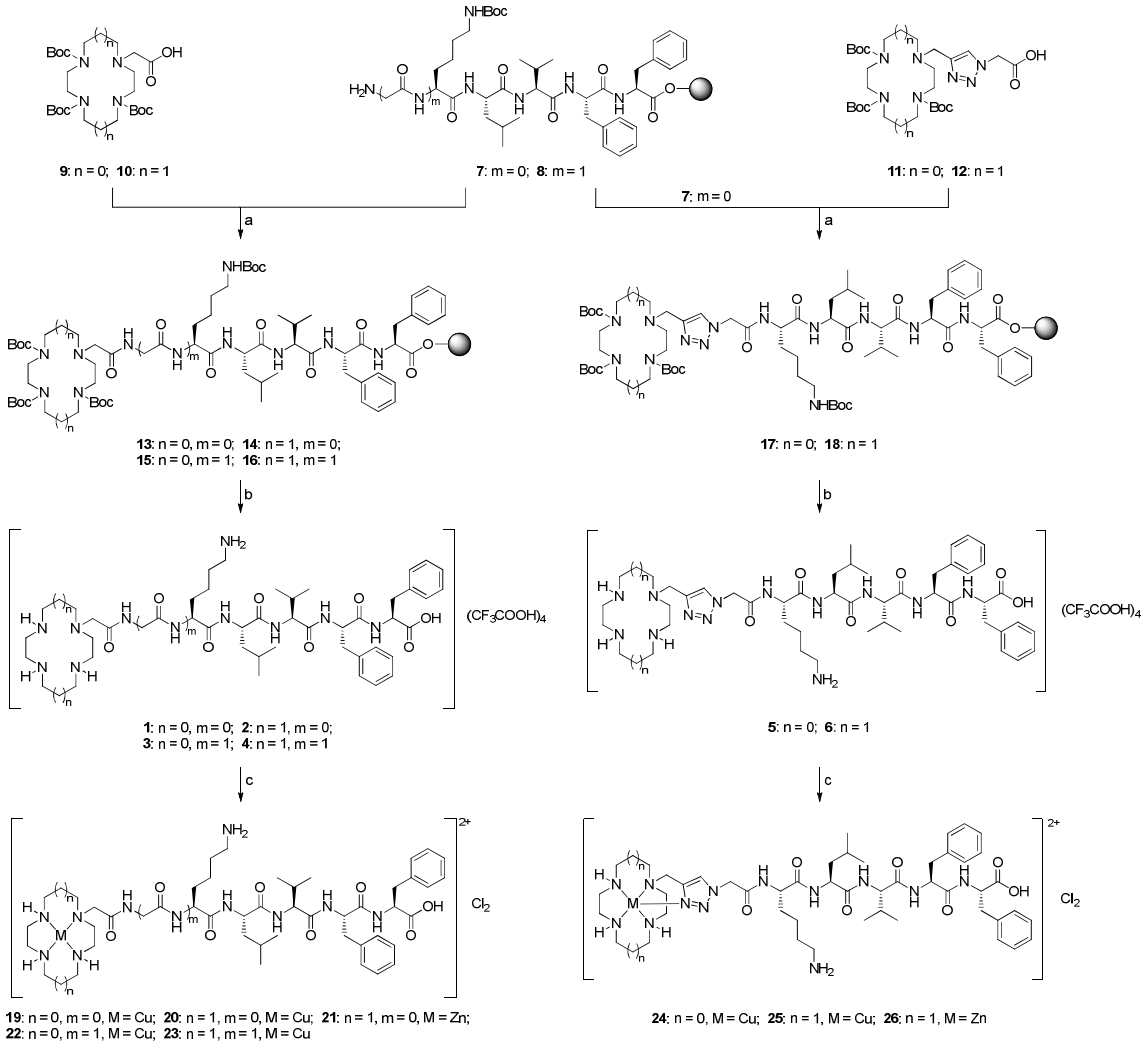
Propargyl-tri-Boc cyclen **S10** (1.02 g, 2.00 mmol) and 2-azidoacetic acid[25] (0.202 g, 2.00 mmol) were dissolved in *t*-BuOH/H_2O (1:1, 40 mL). A brown cloudy solution of $CuSO_4{\cdot}5H_2O$ (25 mg, 0.10 mmol, 5 mol%) and sodium ascorbate (40 mg, 0.20 mol, 10 mol%) in H_2O (4 mL) was added. The reaction mixture was stirred under Ar at room temperature overnight, quenched with 5% $NaHCO_3$ (10 mL), taken to pH 4-5 with 10% citric acid and extracted with EtOAc (3 × 80 mL). The combined organic extracts were concentrated under reduced pressure, and the residue was purified by flash column chromatography (silica gel, EtOAc ramping to EtOAc:CH_3OH = 7:3) to give the **11** as a white foam (1.22 g, 100%). $\boldsymbol{R_F}$ (EtOAc:CH_3OH = 9:1) 0.13. **IR** ν_{max}/cm^{-1} 3478, 2974, 2932, 2827, 1679, 1462, 1413, 1364, 1247, 1156, 1048, 772. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.44 (s, 18H, 2 × C(CH_3)$_3$), 1.46 (s, 9H, C(CH_3)$_3$), 2.75-2.95 (m, 4H, CH_2N(CH_2-triazole)CH_2), 3.25-3.65 (br m, 12H, 3 × CH_2N(Boc)CH_2), 4.05 (br s, 2H, NCH_2-triazole), 5.10 (s, 2H, triazole-CH_2COOH), 6.48 (br s, 1H, COOH), 7.80 (br s, 1H, triazole-H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 28.2, 28.4, 45.3, 46.7, 47.8, 49.4, 51.3, 52.0, 79.6, 79.9, 125.8, 140.0, 155.5, 155.9, 168.9 (thirteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 610.2 ([M-H]$^-$, 100%), 1221.5 ([2M-H]$^-$, 55%). **HRMS** (ESI) 612.37210 ([M+H]$^+$); calcd. for $C_{28}H_{50}N_7O_8$ ([M+H]$^+$) 612.37154.

2-(4-((4,8,11-Tris(*tert*-butoxycarbonyl)-1,4,8,11-tetraazacyclotetradecan-1-yl)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid (12).

Propargyl-tri-Boc cyclam **S11** (1.08 g, 2.00 mmol) and 2-azidoacetic acid[25] (0.203 g, 2.01 mmol) were dissolved in *t*-BuOH/H_2O (1:1, 40 mL). A brown cloudy solution of $CuSO_4{\cdot}5H_2O$ (25 mg, 0.10 mmol, 5 mol%) and sodium ascorbate (40 mg, 0.20 mol, 10 mol%) in H_2O (4 mL) was added. The reaction mixture was stirred under Ar at room temperature overnight, quenched with saturated NH_4Cl (10 mL) and extracted with EtOAc (3 × 80 mL). The combined organic extracts were concentrated under reduced

pressure, and the residue was purified by flash column chromatography (silica gel, EtOAc ramping to EtOAc:CH_3OH = 7:3) to give the **12** as a white foam (1.26 g, 98%). $\boldsymbol{R_F}$ (EtOAc:CH_3OH = 9:1) 0.13. **IR** ν_{max}/cm^{-1} 3454, 2974, 2934, 2108, 1684, 1626, 1468, 1413, 1370, 1302, 1241, 1157, 1055, 734. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 1.43 (s, 18H, 2 × $C(CH_3)_3$), 1.46 (s, 9H, $C(CH_3)_3$), 1.70-1.83 (m, 2H, $CH_2CH_2CH_2$), 1.83-2.00 (m, 2H, $CH_2CH_2CH_2$), 2.50-2.70 (m, 2H, $CH_2N(CH_2$-triaozle$)CH_2$), 2.70-2.90 (m, 2H, $CH_2N(CH_2$-triazole$)CH_2$), 3.15-3.55 (m, 12H, 3 × $CH_2N(Boc)CH_2$), 3.93 (br s, 2H, NCH_2-triazole), 4.96 (s, 2H, triazole-CH_2COOH), 7.09 (br s, 1H, COOH), 7.76 (br s, 1H, triazole-H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 25.2, 28.4, 45.3, 46.4, 46.9, 47.3, 48.3, 50.4, 51.4, 52.8, 79.6, 79.8, 125.5, 140.8, 155.4, 155.7, 171.5 (thirteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 638.3 ($[M-H]^-$, 100%), 1277.5 ($[2M-H]^-$, 48%). **HRMS** (ESI) 662.38603 ($[M+Na]^+$); calcd. for $C_{30}H_{53}N_7NaO_8$ ($[M+Na]^+$) 662.38478.

5. Synthesis of Tetraazamacrocycle-(G)KLVFF Hybrids 1-6 and Metal Complexes 19-26



Scheme S3. Synthesis of tetraazamacrocycle-(G)KLVFF hybrids **1**-**6** and their metal complexes **19-26**. Reagents and conditions: (a) appropriate carboxylic acid (**9**, **10**, **11** or **12**), PyBOP, NMM, DMF, rt, 1 h; (b) TFA/TIS/H_2O (90:5:5), rt, 2 h, followed by RP-HPLC purification, **1**: 53%, **2**: 63%, **3**: 52%, **4**: 60%, **5**: 60%, **6**: 58%; (c) $CuCl_2 \cdot 2H_2O$ or $ZnCl_2$, EtOH, reflux, 6 h, **19**: 94%, **20**: 81%, **21**: 54%, **22**: 85%, **23**: 88%, **24**: 67%, **25**: 53%, **26**: 69%.

10-((4*S*,7*S*,10*S*,13*S*,16*S*)-4-(4-Ammoniobutyl)-13-benzyl-16-carboxy-7-isobutyl-10-isopropyl-2,5,8,11,14-pentaoxo-17-phenyl-3,6,9,12,15-pentaazaheptadecyl)-10-aza-1,4,7-triazoniacyclododecane-1,4,7-triium 2,2,2-trifluoroacetate (1).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 227 mg, 0.250 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclen-pentapeptide conjugate **1** was assembled using

general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 50% B over 45 min) to give **1** as a white solid (174 mg, 53%). **m.p.** 169-170 °C. **$[\alpha]_D^{20}$** -42.6 (*c* 1.0, H_2O). **IR** ν_{max}/cm^{-1} 3273, 3074, 2961, 2871, 1672, 1630, 1539, 1420, 1362, 1184, 1131, 707. **^{1}H NMR** (400 MHz, D_2O) δ 0.68 (d, 3H, *J* 6.4, CH_3), 0.76 (d, 3H, *J* 7.2, CH_3), 0.78 (d, 3H, *J* 6.4, CH_3), 0.84 (d, 3H, *J* 6.0, CH_3), 1.25-1.44 (m, 3H), 1.44-1.55 (m, 2H), 1.55-1.65 (m, 2H), 1.65-1.77 (m, 2H), 1.77-1.90 (m, 1H) (total 10H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NH$_3^+$ & C*H*$_2$C*H*(CH$_3$)$_2$ & CHC*H*(CH$_3$)$_2$), 2.70-2.84 (m, 2H), 2.84-3.03 (m, 10H), 3.03-3.30 (m, 10H) (total 22H, 2 × C*H*$_2$Ph & C*H*$_2$NH$_3^+$ & 3 × C*H*$_2$NH$_2^+$C*H*$_2$ & C*H*$_2$N(CH$_2$CONH)C*H*$_2$), 3.44 (s, 2H, NC*H*$_2$CONH), 4.04 (d, 1H, *J* 8.0, NHC*H*CO), 4.23 (t, 1H, *J* 6.8, NHC*H*CO), 4.28-4.38 (m, 1H, NHC*H*CO), 4.52-4.64 (m, 2H, 2 × NHC*H*CO), 7.08 (d, 2H, *J* 7.2, Ph-H), 7.12 (d, 2H, *J* 7.2, Ph-H), 7.14-7.25 (m, 6H, Ph-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), five amide proton signals (5 × CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (100 MHz, D_2O) δ 17.7, 18.5, 21.2, 22.0, 24.3, 26.4, 30.6, 30.8, 36.7, 37.6, 39.1, 39.8, 42.1, 42.5, 44.3, 49.6, 52.3, 53.8, 53.9, 54.4, 55.1, 59.0, 116.3 (q, $J_{C\text{-}F}$ 290.0, 4 × CF_3), 127.1, 128.6, 129.1, 129.2, 136.1, 136.3, 162.7 (q, $J_{C\text{-}F}$ 40.0, 4 × CF_3*C*OOH), 172.0, 172.1, 173.1, 173.4, 173.6, 174.0 (eleven carbon signals overlapping or obscured). **MS** (ESI) *m/z* 866.0 ([M-4TFA+H]$^+$, 100%). **HRMS** (ESI) 865.56469 ([M-4TFA+H]$^+$); calcd. for $C_{45}H_{73}N_{10}O_7$ ([M-4TFA+H]$^+$) 865.56582. **Anal.** Calcd. for $C_{53}H_{76}F_{12}N_{10}O_{15}$: C 48.18, H 5.80, N 10.60; Found: C 48.44, H 6.06, N 10.82.

11-((4*S*,7*S*,10*S*,13*S*,16*S*)-4-(4-Ammoniobutyl)-13-benzyl-16-carboxy-7-isobutyl-10-isopropyl-2,5,8,11,14-pentaoxo-17-phenyl-3,6,9,12,15-pentaazaheptadecyl)-11-aza-1,4,8-triazoniacyclotetradecane-1,4,8-triium 2,2,2-trifluoroacetate (2).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 227 mg, 0.250 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclam-pentapeptide conjugate **2** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 50% B over 45 min) to give **2** as a white solid (213 mg, 63%). **m.p.** 155-156 °C. **$[\alpha]_D^{20}$** -43.4 (*c* 1.0, H_2O). **IR** ν_{max}/cm^{-1} 3272, 3074, 2959, 2865, 1672, 1628, 1544, 1428, 1364, 1185, 1128, 833, 797, 706. **^{1}H NMR** (400 MHz, D_2O) δ 0.69 (d,

3H, *J* 6.4, CH_3), 0.77 (d, 3H, *J* 7.2, CH_3), 0.79 (d, 3H, *J* 5.6, CH_3), 0.84 (d, 3H, *J* 5.2, CH_3), 1.26-1.54 (m, 5H), 1.54-1.66 (m, 2H), 1.66-1.76 (m, 2H), 1.76-2.10 (m, 5H) (total 14H, 2 × $NCH_2CH_2CH_2N$ & $CH_2CH_2CH_2CH_2NH_3^+$ & $CH_2CH(CH_3)_2$ & $CHCH(CH_3)_2$), 2.60-3.50 (br m, 24H, 2 × CH_2Ph & $CH_2NH_3^+$ & 3 × $CH_2NH_2^+CH_2$ & $CH_2N(CH_2CONH)CH_2$), 4.07 (d, 1H, *J* 8.0, NHC*H*CO), 4.24 (t, 1H, *J* 6.8, NHC*H*CO), 4.28-4.36 (m, 1H, NHC*H*CO), 4.54-4.63 (m, 2H, 2 × NHC*H*CO), 7.09 (d, 2H, *J* 7.2, Ph-H), 7.14 (d, 2H, *J* 7.2, Ph-H), 7.15-7.26 (m, 6H, Ph-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), five amide proton signals (5 × CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, D_2O) δ 17.8, 18.5, 21.4, 22.0, 22.5, 23.5, 24.3, 26.4, 30.7, 36.7, 37.7, 39.0, 40.2, 42.9, 44.5, 45.5, 46.7, 52.2, 53.5, 53.9, 54.3, 54.6, 58.9, 116.3 (q, $J_{C\text{-}F}$ 292.5, 4 × CF_3), 127.0, 128.5, 129.0, 129.1, 136.1, 136.3, 162.6 (q, $J_{C\text{-}F}$ 37.5, 4 × CF_3CCOOH), 171.9, 173.2, 173.4, 173.9 (fourteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 447.3 ($[M\text{-}4TFA+2H]^{2+}$, 56%), 893.6 ($[M\text{-}4TFA+H]^+$, 100%). **HRMS** (ESI) 893.59554 ($[M\text{-}4TFA+H]^+$); calcd. for $C_{47}H_{77}N_{10}O_7$ ($[M\text{-}4TFA+H]^+$) 893.59712. **Anal.** Calcd. for $C_{55}H_{80}F_{12}N_{10}O_{15}\cdot H_2O$: C 48.31, H 6.04, N 10.24; Found: C 48.40, H 6.07, N 10.42.

10-((7*S*,10*S*,13*S*,16*S*,19*S*)-7-(4-Ammoniobutyl)-16-benzyl-19-carboxy-10-isobutyl-13-isopropyl-2,5,8,11,14,17-hexaoxo-20-phenyl-3,6,9,12,15,18-hexaazaicosyl)-10-aza-1,4,7-triazoniacyclododecane-1,4,7-triium 2,2,2-trifluoroacetate (3).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 227 mg, 0.250 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclen-hexapeptide conjugate **3** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 50% B over 45 min) to give **3** as a white solid (179 mg, 52%). **m.p.** 216-217 °C. **$[\alpha]_D^{20}$** -41.0 (*c* 0.50, H_2O). **IR** ν_{max}/cm^{-1} 3270, 3075, 2962, 2874, 1674, 1627, 1531, 1423, 1363, 1185, 1131, 834, 796, 717. **^{1}H NMR** (400 MHz, D_2O) δ 0.72 (d, 3H, *J* 6.8, CH_3), 0.80 (d, 3H, *J* 6.8, CH_3), 0.83 (d, 3H, *J* 6.4, CH_3), 0.90 (d, 3H, *J* 6.0, CH_3), 1.28-1.48 (m, 3H), 1.48-1.59 (m, 2H), 1.59-1.69 (m, 2H), 1.69-1.81 (m, 2H), 1.81-1.93 (m, 1H) (total 10H, $CH_2CH_2CH_2CH_2NH_3^+$ & $CH_2CH(CH_3)_2$ & $CHCH(CH_3)_2$), 2.70-3.30 (br m, 22H, 2 × CH_2Ph & $CH_2NH_3^+$ & 3 × $CH_2NH_2^+CH_2$ & $CH_2N(CH_2CONH)CH_2$), 3.50 (s, 2H,

NCH_2CONH), 3.93-4.03 (m, 3H, NHCHCO & CONHCH_2CONH), 4.25 (t, 1H, J 6.8, NHCHCO), 4.28-4.32 (m, 1H, NHCHCO), 4.58 (t, 1H, J 9.2, NHCHCO), 4.59 (t, 1H, J 8.8, NHCHCO), 7.17 (d, 2H, J 6.8, Ph-H), 7.21 (d, 2H, J 7.2, Ph-H), 7.25-7.35 (m, 6H, Ph-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), six amide proton signals (6 × CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, D_2O) δ 17.9, 18.6, 21.6, 22.0, 22.2, 24.4, 26.4, 31.1, 31.3, 36.9, 38.0, 39.1, 40.5, 42.1, 42.6, 44.3, 49.6, 51.8, 53.2, 53.8, 54.1, 55.3, 58.6, 116.3 (q, $J_{C\text{-}F}$ 292.5, 4 × CF_3), 126.9, 128.4, 129.1, 136.1, 136.2, 162.5 (q, $J_{C\text{-}F}$ 37.5, 4 × CF_3COOH), 170.3, 171.7, 172.0, 172.5, 173.0, 173.6, 173.8 (twelve carbon signals overlapping or obscured). **MS** (ESI) m/z 461.8 ([M-4TFA+2H]$^{2+}$, 100%), 922.6 ([M-4TFA+H]$^+$, 85%). **HRMS** (ESI) 922.58649 ([M-4TFA+H]$^+$); calcd. for $C_{47}H_{76}N_{11}O_8$ ([M-4TFA+H]$^+$) 922.58728. **Anal.** Calcd. for $C_{55}H_{79}F_{12}N_{11}O_{16}$: C 47.93, H 5.78, N 11.18; Found: C 47.90, H 6.05, N 11.33.

11-((7*S*,10*S*,13*S*,16*S*,19*S*)-7-(4-Ammoniobutyl)-16-benzyl-19-carboxy-10-isobutyl-13-isopropyl-2,5,8,11,14,17-hexaoxo-20-phenyl-3,6,9,12,15,18-hexaazaicosyl)-11-aza-1,4,8-triazoniacyclotetradecane-1,4,8-triium 2,2,2-trifluoroacetate (4).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 227 mg, 0.250 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclam-hexapeptide conjugate **4** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 50% B over 45 min) to give **4** as a white solid (211 mg, 60%). **m.p.** 156-157 °C. **[α]$_D^{20}$** -40.4 (c 1.0, H_2O). **IR** ν_{max}/cm^{-1} 3272, 3074, 3033, 2960, 2866, 1672, 1628, 1535, 1430, 1364, 1187, 1130, 835, 798, 717. **^{1}H NMR** (400 MHz, D_2O) δ 0.72 (d, 3H, J 6.8, CH_3), 0.80 (d, 3H, J 6.8, CH_3), 0.83 (d, 3H, J 6.0, CH_3), 0.90 (d, 3H, J 6.0, CH_3), 1.28-1.47 (m, 3H), 1.47-1.59 (m, 2H), 1.59-1.80 (m, 4H), 1.80-2.00 (m, 5H) (total 14H, 2 × $NCH_2CH_2CH_2N$ & $CH_2CH_2CH_2CH_2NH_3^+$ & $CH_2CH(CH_3)_2$ & $CHCH(CH_3)_2$), 2.70-3.26 (br m, 22H, 2 × CH_2Ph & $CH_2NH_3^+$ & 3 × $CH_2NH_2^+CH_2$ & $CH_2N(CH_2CONH)CH_2$), 3.34 (br s, 2H, NCH_2CONH), 3.95-4.06 (m, 1H, NHCHCO), 3.99 (s, 2H, CONHCH_2CONH), 4.23-4.30 (m, 2H, 2 × NHCHCO), 4.58 (t, 1H, J 9.2, NHCHCO), 4.59 (t, 1H, J 8.8, NHCHCO), 7.16 (d, 2H, J 6.8, Ph-H), 7.20 (d, 2H, J 7.2, Ph-H), 7.25-7.34 (m, 6H, Ph-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), six amide proton signals (6 × CONH) and one

carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, D_2O) δ 17.7, 18.4, 21.0, 22.0, 22.6, 24.0, 24.3, 26.4, 30.5, 30.8, 36.7, 37.5, 39.1, 39.7, 41.9, 43.3, 44.8, 46.0, 46.9, 47.3, 52.3, 53.6, 53.9, 54.4, 54.8, 59.0, 116.3 (q, $J_{C\text{-}F}$ 292.5, 4 × CF_3), 127.2, 128.6, 128.7, 129.1, 129.2, 136.1, 136.3, 162.8 (q, $J_{C\text{-}F}$ 37.5, 4 × CF_3*C*OOH), 170.9, 172.0, 172.2, 173.4, 173.7, 174.1 (ten carbon signals overlapping or obscured). **MS** (ESI) *m/z* 475.8 ([M-4TFA+2H]$^{2+}$, 100%), 950.6 ([M-4TFA+H]$^+$, 65%). **HRMS** (ESI) 950.61653 ([M-4TFA+H]$^+$); calcd. for $C_{49}H_{80}N_{11}O_8$ ([M-4TFA+H]$^+$) 950.61859. **Anal.** Calcd. for $C_{57}H_{83}F_{12}N_{11}O_{16}$: C 48.68, H 5.95, N 10.96; Found: C 48.47, H 6.19, N 11.05.

10-((1-((4*S*,7*S*,10*S*,13*S*,16*S*)-4-(4-Ammoniobutyl)-13-benzyl-16-carboxy-7-isobutyl-10-isopropyl-2,5,8,11,14-pentaoxo-17-phenyl-3,6,9,12,15-pentaazaheptadecyl)-1*H*-1,2,3-triazol-4-yl)methyl)-10-aza-1,4,7-triazoniacyclododecane-1,4,7-triium 2,2,2-trifluoroacetate (5).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 227 mg, 0.250 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclen-pentapeptide conjugate **5** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 40% B over 45 min) to give **5** as a white solid (209 mg, 60%). **m.p.** 217-218 °C. **$[\alpha]_D^{20}$** -44.6 (*c* 1.0, H_2O). **IR** ν_{max}/cm^{-1} 3274, 3078, 2961, 2869, 1672, 1630, 1546, 1421, 1363, 1186, 1131, 833, 798, 706. **^{1}H NMR** (400 MHz, D_2O) δ 0.73 (d, 3H, *J* 6.4, CH_3), 0.80 (d, 3H, *J* 6.8, CH_3), 0.82 (d, 3H, *J* 6.0, CH_3), 0.89 (d, 3H, *J* 6.0, CH_3), 1.33-1.49 (m, 3H), 1.49-1.62 (m, 2H), 1.62-1.74 (m, 2H), 1.74-1.93 (m, 3H) (total 10H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$NH$_3^+$ & C*H*$_2$C*H*(CH$_3$)$_2$ & CHC*H*(CH$_3$)$_2$), 2.70-3.50 (br m, 22H, 2 × C*H*$_2$Ph & C*H*$_2$NH$_3^+$ & 3 × C*H*$_2$NH$_2^+$C*H*$_2$ & C*H*$_2$N(CH$_2$-triazole)C*H*$_2$), 3.95 (s, 2H, CH$_2$N(C*H*$_2$-triazole)CH$_2$), 4.02 (d, 1H, *J* 8.0, NHC*H*CO), 4.29-4.34 (m, 2H, 2 × NHC*H*CO), 4.57-4.65 (m, 2H, 2 × NHC*H*CO), 5.32 (s, 2H, triazole-C*H*$_2$CONH), 7.19 (d, 2H, *J* 6.8, Ph-H), 7.24 (d, 2H, *J* 7.2, Ph-H), 7.27-7.37 (m, 6H, Ph-H), 8.02 (s, 1H, triazole-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), five amide proton signals (5 × CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, D_2O) δ 17.7, 18.4, 20.8, 22.1, 24.3, 26.3, 30.4, 36.7, 37.4, 39.1, 39.6, 41.7, 42.0, 44.3, 46.3, 47.6, 51.7, 52.3, 53.9, 54.4, 59.0, 116.3 (q, $J_{C\text{-}F}$ 292.5, 4 × CF_3), 126.6, 127.2, 128.7, 129.1, 129.2, 136.2, 136.3, 142.4, 162.8 (q, $J_{C\text{-}F}$ 37.5, 4 × CF_3*C*OOH), 167.6, 172.1, 172.2, 173.2, 173.8,

174.1 (thirteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 473.5 ([M-4TFA+2H]$^{2+}$, 100%), 946.5 ([M-4TFA+H]$^{+}$, 5%). **HRMS** (ESI) 946.59841 ([M-4TFA+H]$^{+}$); calcd. for $C_{48}H_{76}N_{13}O_7$ ([M-4TFA+H]$^{+}$) 946.59852. **Anal.** Calcd. for $C_{56}H_{79}F_{12}N_{13}O_{15}$: C 47.96, H 5.68, N 12.99; Found: C 47.95, H 5.99, N 13.26.

11-((1-((4*S*,7*S*,10*S*,13*S*,16*S*)-4-(4-Ammoniobutyl)-13-benzyl-16-carboxy-7-isobutyl-10-isopropyl-2,5,8,11,14-pentaoxo-17-phenyl-3,6,9,12,15-pentaazaheptadecyl)-1*H*-1,2,3-triazol-4-yl)methyl)-11-aza-1,4,8-triazoniacyclotetradecane-1,4,8-triium 2,2,2-trifluoroacetate (6).

Wang resin (100-200 mesh, loading 1.1 mmol/g, 182 mg, 0.200 mmol) was pre-loaded with Fmoc-Phe-OH (**S1**) and cyclam-pentapeptide conjugate **6** was assembled using general synthetic procedure A. The combined cleavage solution and TFA washings were concentrated under reduced pressure, and the residue was purified by preparative RP-HPLC (gradient 0% to 40% B over 45 min) to give **6** as a white solid (165 mg, 58%). **m.p.** 150-151 °C. **[α]$_D^{20}$** -42.3 (*c* 1.0, H_2O). **IR** ν_{max}/cm^{-1} 3273, 3076, 2961, 2868, 1672, 1630, 1547, 1430, 1366, 1187, 1131, 836, 799, 717. **^{1}H NMR** (400 MHz, D_2O) δ 0.73 (d, 3H, *J* 6.4, CH_3), 0.81 (d, 3H, *J* 7.6, CH_3), 0.82 (d, 3H, *J* 6.8, CH_3), 0.90 (d, 3H, *J* 5.6, CH_3), 1.33-1.50 (m, 3H), 1.50-1.63 (m, 2H), 1.63-1.75 (m, 2H), 1.75-1.96 (m, 5H), 2.00-2.15 (m, 2H) (total 14H, 2 × $NCH_2CH_2CH_2N$ & $CH_2CH_2CH_2CH_2NH_3^+$ & $CH_2CH(CH_3)_2$ & $CHCH(CH_3)_2$), 2.70-3.40 (br m, 22H, 2 × CH_2Ph & $CH_2NH_3^+$ & 3 × $CH_2NH_2^+CH_2$ & $CH_2N(CH_2\text{-triazole})CH_2$), 3.82 (br s, 2H, $CH_2N(CH_2\text{-triazole})CH_2$), 4.02 (d, 1H, *J* 8.0, NHC*H*CO), 4.28-4.33 (m, 2H, 2 × NHC*H*CO), 4.57-4.65 (m, 2H, 2 × NHC*H*CO), 5.33 (s, 2H, triazole-CH_2CONH), 7.19 (d, 2H, *J* 7.2, Ph-H), 7.24 (d, 2H, *J* 7.2, Ph-H), 7.27-7.37 (m, 6H, Ph-H), 7.97 (s, 1H, triazole-H) (nine ammonium proton signals (3 × NH_2^+ & NH_3^+), five amide proton signals (5 × CONH) and one carboxylic acid proton signal (COOH) not observed due to H/D exchange). **^{13}C NMR** (75 MHz, D_2O) δ 17.5, 18.2, 20.7, 21.9, 24.1, 26.2, 30.3, 36.6, 37.3, 39.0, 39.4, 39.6, 41.2, 41.5, 43.3, 43.6, 46.6, 48.0, 50.5, 51.6, 52.1, 53.7, 54.3, 58.9, 115.8 (q, $J_{C\text{-}F}$ 360.0, 4 × CF_3), 127.0, 128.5, 129.0, 136.0, 136.2, 141.0, 161.7 (q, $J_{C\text{-}F}$ 60.0, 4 × CF_3COOH), 167.3, 171.9, 172.0, 173.0, 173.6, 174.0 (fourteen carbon signals overlapping or obscured). **MS** (ESI) *m/z* 487.5 ([M-4TFA+2H]$^{2+}$, 100%), 974.6 ([M-4TFA+H]$^{+}$, 10%). **HRMS** (ESI) 974.63091 ([M-4TFA+H]$^{+}$); calcd. for

$C_{50}H_{80}N_{13}O_7$ ([M-4TFA+H]$^+$) 974.62982. **Anal.** Calcd. for $C_{58}H_{83}F_{12}N_{13}O_{15}\cdot 2H_2O$: C 47.51, H 5.98, N 12.42; Found: C 47.43, H 5.76, N 12.50.

[Cu(1-4TFA)]Cl_2 complex (19).

Compound **1** (119 mg, 0.0900 mmol) and $CuCl_2\cdot 2H_2O$ (15.3 mg, 0.0897 mmol) were complexed according to general synthetic procedure B to give **19** as a blue powder (85.1 mg, 94%). **m.p.** 170-175 °C. **[α]$_D^{20}$** -53.0 (*c* 0.10, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 586, ε 211. **IR** ν_{max}/cm^{-1} 3411, 3269, 3082, 2957, 1632, 1546, 1456, 1396, 1199, 1136, 1080, 700. **HRMS** (ESI) 463.74372, 464.24552, 464.74318, 465.24474, 465.74642, 466.24827 ([M-2Cl]$^{2+}$); calcd. for $C_{45}H_{72}CuN_{10}O_7$ ([M-2Cl]$^{2+}$) 463.74352, 464.24516, 464.74284, 465.24432, 465.74597, 466.24765. **Anal.** Calcd. for $C_{45}H_{72}Cl_2CuN_{10}O_7\cdot CF_3COOH\cdot 2H_2O$: C 49.10, H 6.75, N 12.18; Found: C 49.01, H 6.61, N 12.19.

[Cu(2-4TFA)]Cl_2 complex (20).

Compound **2** (135 mg, 0.100 mmol) and $CuCl_2\cdot 2H_2O$ (17.1 mg, 0.100 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (10 mL), washed with CH_3CN (3 × 10 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* to give **20** as a purple powder (83.3 mg, 81%). **m.p.** 160-165 °C. **[α]$_D^{20}$** -66.5 (*c* 0.20, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 555, ε 138. **IR** ν_{max}/cm^{-1} 3272, 3076, 2934, 2879, 1633, 1540, 1452, 1395, 1192, 1132, 1040, 699. **HRMS** (ESI) 477.75911, 478.26068, 478.75802, 479.25963, 479.76098 ([M-2Cl]$^{2+}$); calcd. for $C_{47}H_{76}CuN_{10}O_7$ ([M-2Cl]$^{2+}$) 477.75917, 478.26081, 478.75850, 479.25998, 479.76162. **Anal.** Calcd. for $C_{47}H_{76}Cl_2CuN_{10}O_7\cdot CF_3COOH\cdot 3H_2O$: C 49.22, H 7.00, N 11.71; Found: C 48.98, H 6.95, N 11.68.

[Zn(2-4TFA)]Cl_2 complex (21).

Compound **2** (41 mg, 0.030 mmol) and $ZnCl_2$ (4.1 mg, 0.030 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (5 mL), washed with CH_3CN (3 × 5 mL) and Et_2O (3 × 5 mL), and dried *in vacuo* to give **21** as a white powder

(17 mg, 54%). **m.p.** 230-235 °C. **[α]$_D^{20}$** -68.5 (*c* 0.20, H_2O). **IR** ν_{max}/cm^{-1} 3230, 3079, 2936, 2862, 1633, 1524, 1197, 1140, 999, 950, 870, 702. **HRMS** (ESI) 478.25962, 478.76159, 479.25780, 479.75968, 480.25720, 480.75922, 481.26122 ([M-2Cl]$^{2+}$); calcd. for $C_{47}H_{76}N_{10}O_7Zn$ ([M-2Cl]$^{2+}$) 478.25895, 478.76060, 479.25758, 479.75906, 480.25691, 480.75849, 481.26014. **Anal.** Calcd. for $C_{47}H_{76}Cl_2N_{10}O_7Zn{\cdot}4CF_3COOH{\cdot}3CH_3CN{\cdot}4H_2O$: C 43.59, H 5.82, N 10.83; Found: C 43.51, H 6.22, N 10.53.

[Cu(3-4TFA)]Cl$_2$ complex (22).

Compound **3** (110 mg, 0.0798 mmol) and $CuCl_2{\cdot}2H_2O$ (13.6 mg, 0.0798 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (10 mL), washed with 1% EtOH in CH_3CN (3 × 10 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* to give **22** as a blue powder (71.4 mg, 85%). **m.p.** 185-190 °C. **[α]$_D^{20}$** -52.0 (*c* 0.10, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 582, ε 220. **IR** ν_{max}/cm^{-1} 3267, 3086, 2957, 2928, 1627, 1535, 1452, 1399, 1198, 1134, 1078, 698. **HRMS** (ESI) 492.25531, 492.75700, 493.25507, 493.75641, 494.25801, 494.75905 ([M-2Cl]$^{2+}$); calcd. for $C_{47}H_{75}CuN_{11}O_8$ ([M-2Cl]$^{2+}$) 492.25426, 492.75589, 493.25359, 493.75506, 494.25670, 494.75838. **Anal.** Calcd. for $C_{47}H_{75}Cl_2CuN_{11}O_8{\cdot}CF_3COOH{\cdot}2H_2O$: C 48.77, H 6.68, N 12.77; Found: C 48.70, H 6.77, N 12.95.

[Cu(4-4TFA)]Cl$_2$ complex (23).

Compound **4** (141 mg, 0.100 mmol) and $CuCl_2{\cdot}2H_2O$ (17.1 mg, 0.100 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (10 mL), washed with CH_3CN (3 × 10 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* to give **23** as a purple powder (96.2 mg, 88%). **m.p.** 175-180 °C. **[α]$_D^{20}$** -42.5 (*c* 0.20, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 552, ε 110. **IR** ν_{max}/cm^{-1} 3273, 3085, 2956, 2878, 1628, 1539, 1444, 1400, 1191, 1131, 1031, 695. **HRMS** (ESI) 506.27045, 506.77217, 507.27029, 507.77175, 508.27302, 508.77454 ([M-2Cl]$^{2+}$); calcd. for $C_{49}H_{79}CuN_{11}O_8$ ([M-2Cl]$^{2+}$) 506.26991, 506.77154, 507.26925, 507.77071, 508.27235, 508.77403. **Anal.** Calcd. for

$C_{49}H_{79}Cl_2CuN_{11}O_8 \cdot CF_3COOH \cdot 3H_2O$: C 48.90, H 6.92, N 12.30; Found: C 48.59, H 6.86, N 12.30.

[Cu(5-4TFA)]Cl_2 complex (24).

Compound **5** (112 mg, 0.0799 mmol) and $CuCl_2 \cdot 2H_2O$ (13.7 mg, 0.0804 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (10 mL), washed with 1% H_2O in CH_3CN (3 × 10 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* to give **24** as a blue powder (57.9 mg, 67%). **m.p.** 214-215 °C. **$[\alpha]_D^{20}$** -62.5 (*c* 0.080, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 591, ε 258. **IR** ν_{max}/cm^{-1} 3384, 3267, 3080, 2957, 1630, 1545, 1440, 1391, 1203, 1134, 1076, 699. **HRMS** (ESI) 504.26051, 504.76231, 505.26029, 505.76171, 506.26307, 506.76428 ($[M-2Cl]^{2+}$); calcd. for $C_{48}H_{75}CuN_{13}O_7$ ($[M-2Cl]^{2+}$) 504.25987, 504.76150, 505.25921, 505.76067, 506.26232, 506.76400. **Anal.** Calcd. for $C_{48}H_{75}Cl_2CuN_{13}O_7 \cdot 3H_2O$: C 50.81, H 7.20, N 16.05; Found: C 50.65, H 7.12, N 15.93.

[Cu(6-4TFA)]Cl_2 complex (25).

Compound **6** (124 mg, 0.0867 mmol) and $CuCl_2 \cdot 2H_2O$ (14.8 mg, 0.0868 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated under reduced pressure. The residue was triturated with Et_2O (10 mL), washed with 1% H_2O in CH_3CN (3 × 10 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* to give **25** as a purple powder (51.3 mg, 53%). **m.p.** 193-194 °C. **$[\alpha]_D^{20}$** -51.9 (*c* 0.212, H_2O). **UV-Vis** (H_2O) λ_{max}/nm 553, ε 115. **IR** ν_{max}/cm^{-1} 3272, 3082, 2954, 2877, 1668, 1631, 1545, 1455, 1398, 1193, 1138, 1063, 699. **HRMS** (ESI) 518.27644, 518.77816, 519.27609, 519.77749, 520.27905, 520.78054 ($[M-2Cl]^{2+}$); calcd. for $C_{50}H_{79}CuN_{13}O_7$ ($[M-2Cl]^{2+}$) 518.27552, 518.77715, 519.27486, 519.77632, 520.27797, 520.77965. **Anal.** Calcd. for $C_{50}H_{79}Cl_2CuN_{13}O_7 \cdot 5H_2O$: C 50.10, H 7.48, N 15.19; Found: C 50.33, H 7.24, N 15.22.

[Zn(6-4TFA)]Cl_2 complex (26).

Compound **6** (80 mg, 0.056 mmol) and $ZnCl_2$ (7.7 mg, 0.056 mmol) were complexed according to general synthetic procedure B. The reaction mixture was concentrated

under reduced pressure. The residue was triturated with Et_2O (5 mL), washed with CH_3CN (3 × 5 mL) and Et_2O (3 × 5 mL), and dried *in vacuo* to give **26** as a white powder (43 mg, 69%). **m.p.** 230-235 °C. **[α]$_D^{20}$** -52.5 (*c* 0.2, H_2O). **IR** ν_{max}/cm^{-1} 3260, 2944, 1633, 1530, 1192, 1142, 1089, 698, 563. **HRMS** (ESI) 518.77545, 519.27716, 519.77391, 520.27578, 520.77317, 521.27480, 521.77656, 522.27844 ($[M-2Cl]^{2+}$); calcd. for $C_{50}H_{79}N_{13}O_7Zn$ ($[M-2Cl]^{2+}$) 518.77530, 519.27694, 519.77394, 520.27542, 520.77327, 521.27484, 521.77648, 522.27817. **Anal.** Calcd. For $C_{50}H_{79}Cl_2N_{13}O_7Zn{\cdot}4CF_3COOH{\cdot}4CH_3CN{\cdot}4H_2O$: C 43.97, H 5.76, N 13.21; Found: C 44.12, H 6.19, N 13.11.

6. Colors of Copper(II)-Tetraazamacrocycle Complexes 19 and 20

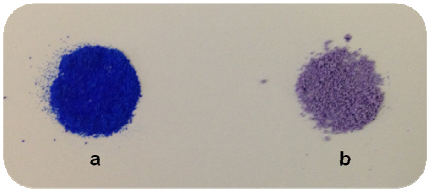
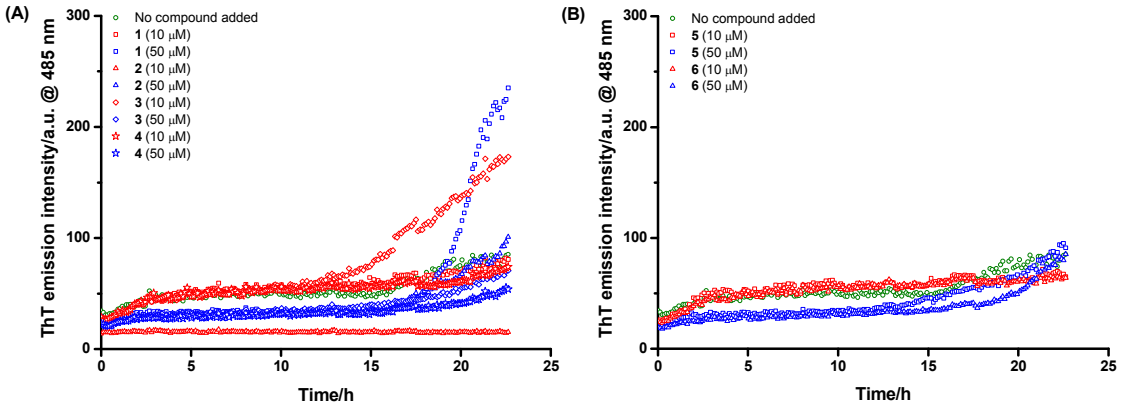


Figure S1. (a) Copper(II)-cyclen-KLVFF complex **19** – a blue powder; (b) Copper(II)-cyclam-KLVFF complex **20** – a purple powder.

7. ThT Extrinsic Fluorescence Assay



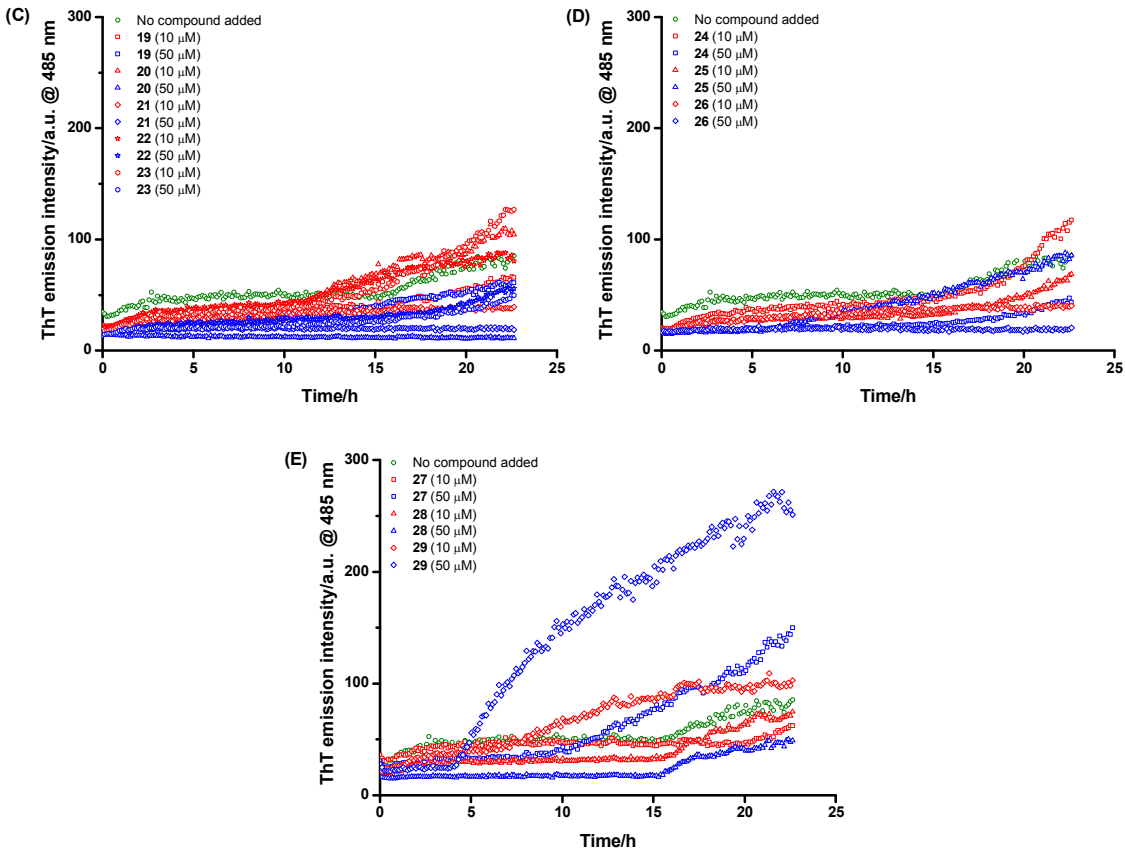


Figure S2. Effects of compounds **1**-**6** and **19**-**29** on Aβ fibril formation. ThT fluorescence over time for Aβ in the absence (green hollow circle) and presence of the amide-tethered hybrids **1**-**4** (A), the triazole-linked hybrids **5** and **6** (B), the amide-tethered metal complexes **19**-**23** (C), the triazole-linked metal complexes **24**-**26** (D) and the control compounds **27**-**29** (E).

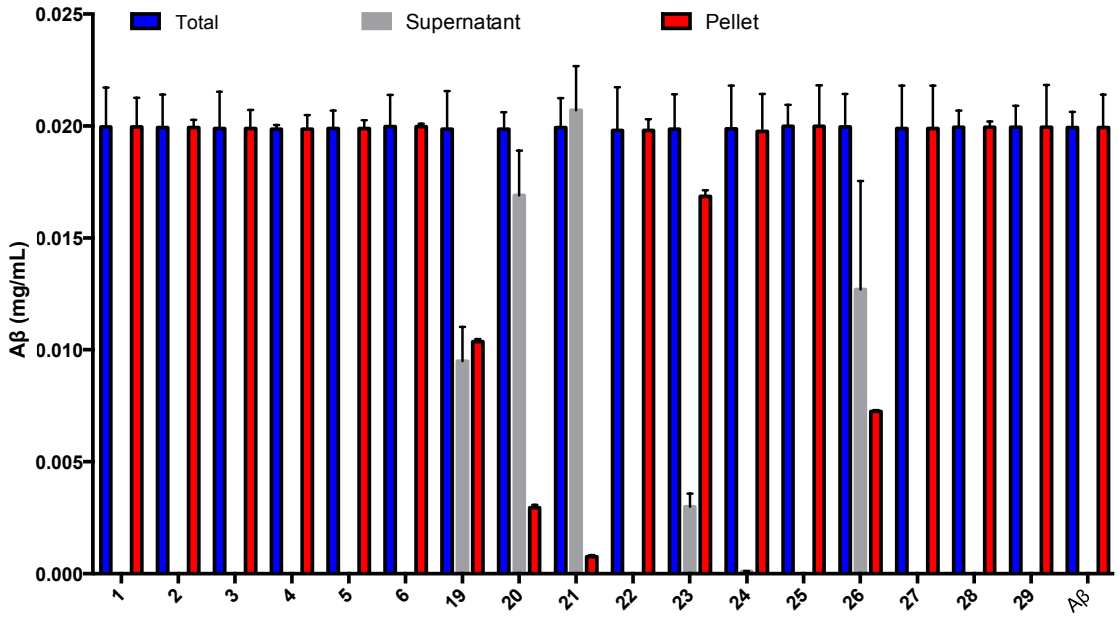


Figure S3. Pelleting assay of $A\beta_{42}$ aggregation. Aβ solutions, incubated for 24 h at 37 °C in the presence and absence of the test compound (50 μM), were centrifuged at 100,000 × *g*. The protein concentration of the sample prior to centrifugation, and of the pellet and supernatant fractions after centrifugation was determined using a microBCA assay, and confirmed using the Direct Detect protein quantitation instrument from Millipore.

8. MALDI-TOF-MS

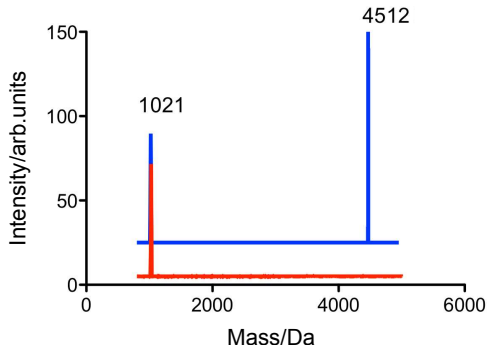
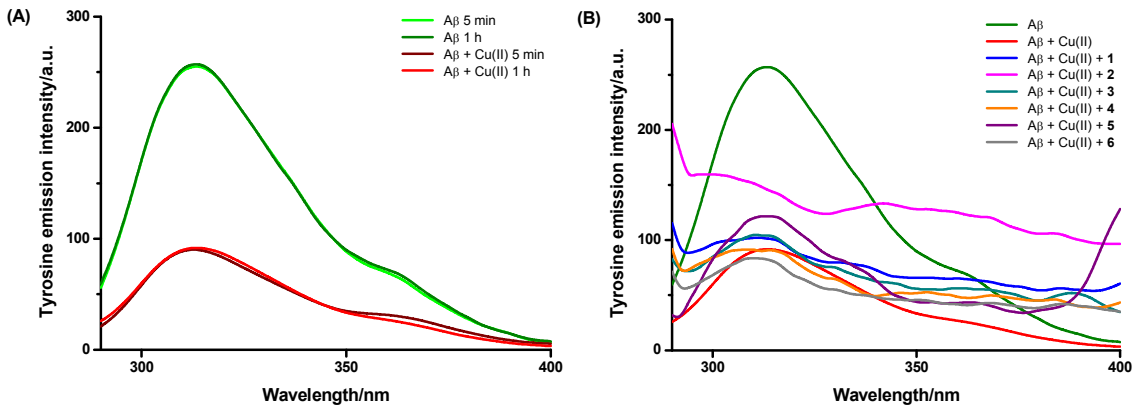
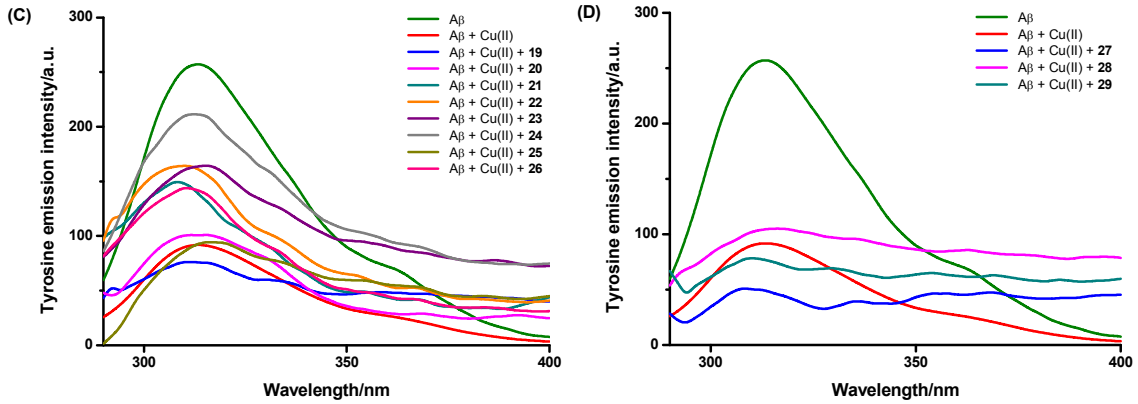
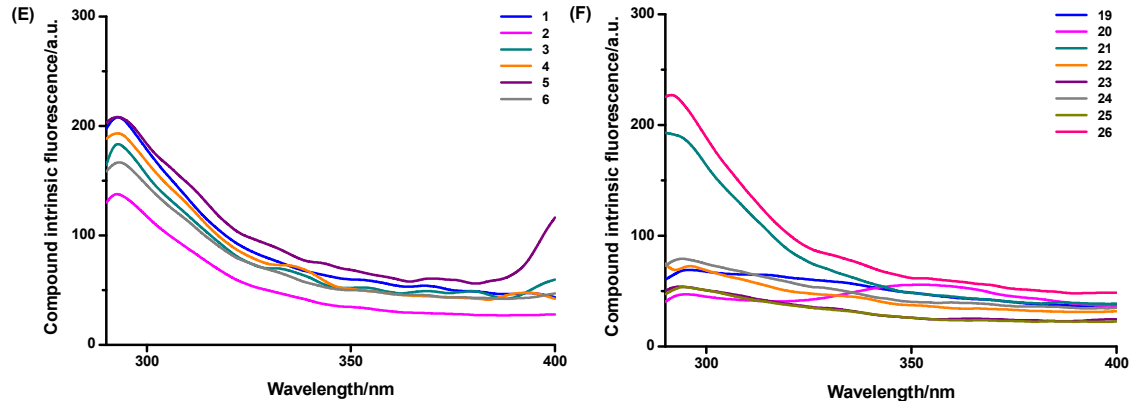


Figure S4. Selected data from MALDI-TOF-MS analysis. MALDI-TOF-MS spectra were recorded from a 7-day incubated solution of compound **22** (50 μM) in PBS buffer (pH 7.4) in the presence (blue signals) or absence (a red signal) of Aβ (10 μM). The signals at *m/z* = 1021 and 4512 indicate the presence of compound **22** and $A\beta_{42}$ respectively.

9. Tyrosine Intrinsic Fluorescence Assay







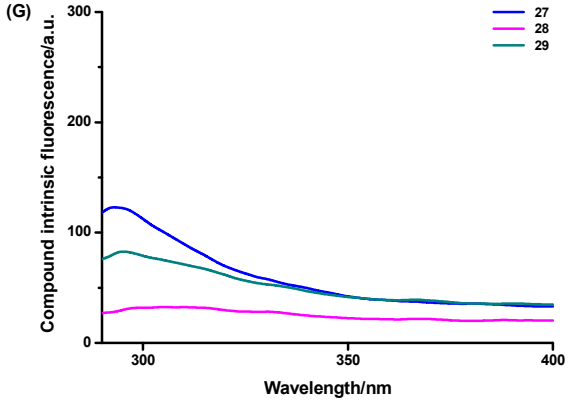


Figure S5. (A) Aβ tyrosine fluorescence over time in the absence and presence of copper (II); (B)-(D) Effects of compounds **1**-**6** and **19**-**29** on the copper(II)-induced quenching of Aβ tyrosine fluorescence. (E)-(G) Intrinsic fluorescence ofcompounds **1**-**6** and **19**-**29**.

10. Bis-ANS Extrinsic Fluorescence Assay

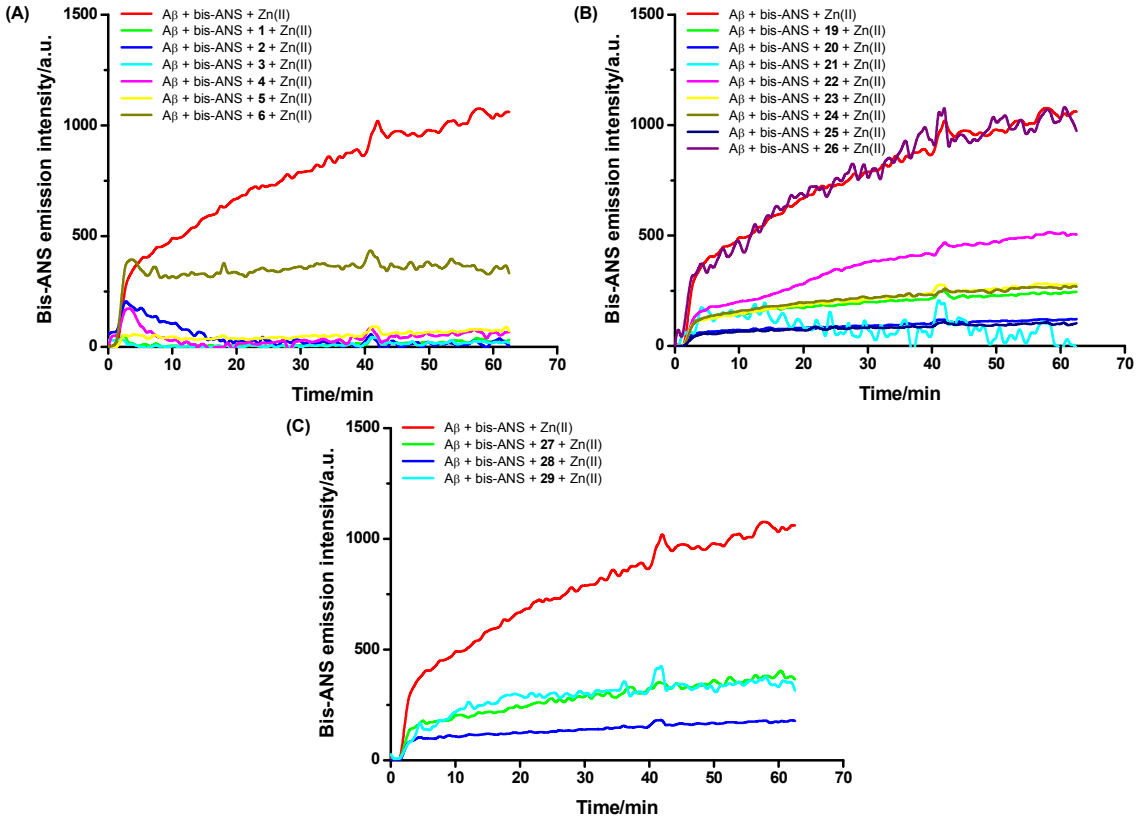


Figure S6. Effects of compounds **1**-**6** and **19**-**29** on the bis-ANS fluorescence intensity in the presence of Aβ and zinc(II).

11. Neurotoxicity Assay

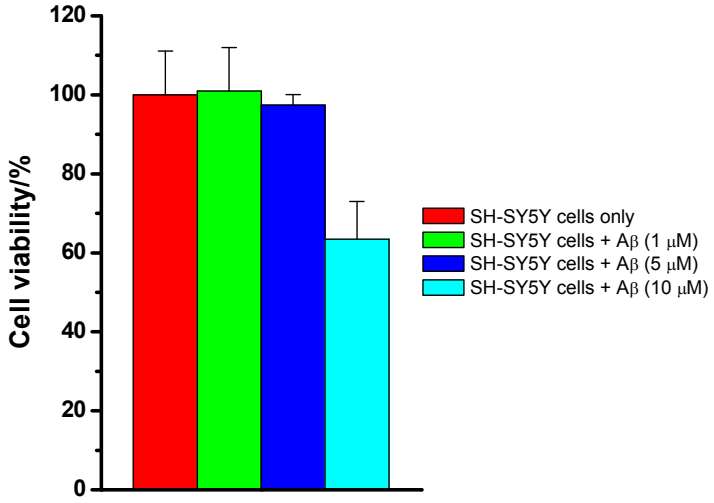


Figure S7. Neurotoxicity of Aβ (0, 1, 5 and 10 μM) against SH-SY5Y cells.

12. ^{1}H & ^{13}C NMR Spectra for Novel Compounds

Figure S8. ^{1}H NMR spectrum (400 MHz) of **11** in $CDCl_3$.

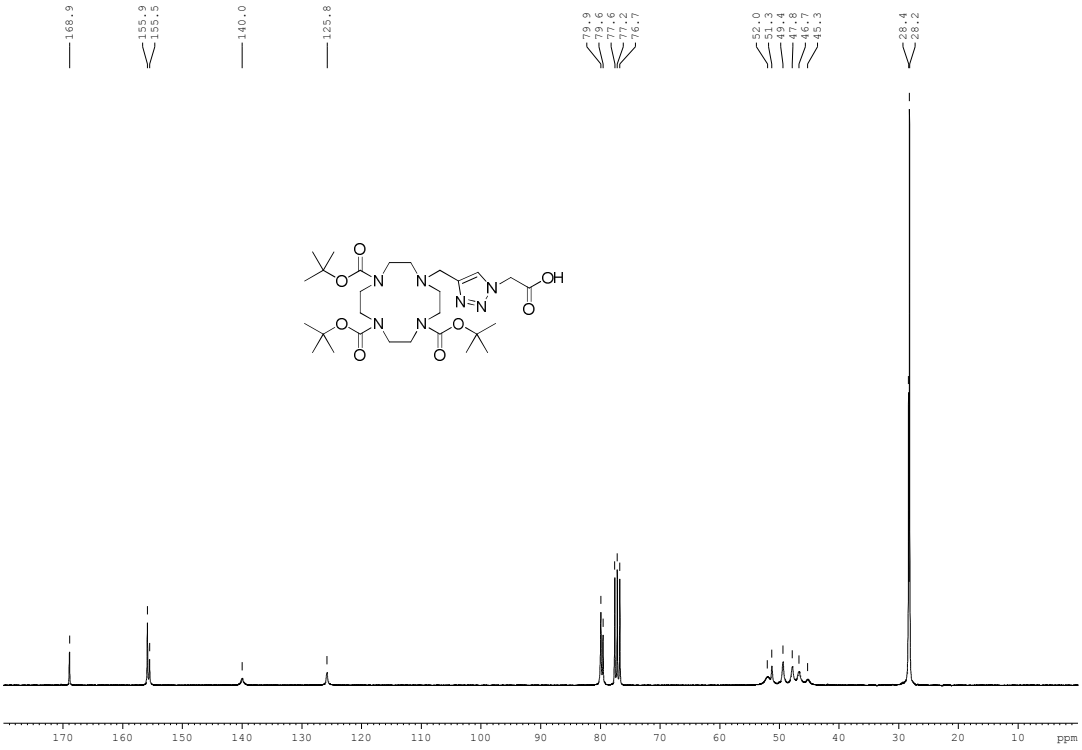


Figure S9. ^{13}C NMR spectrum (75 MHz) of **11** in $CDCl_3$.

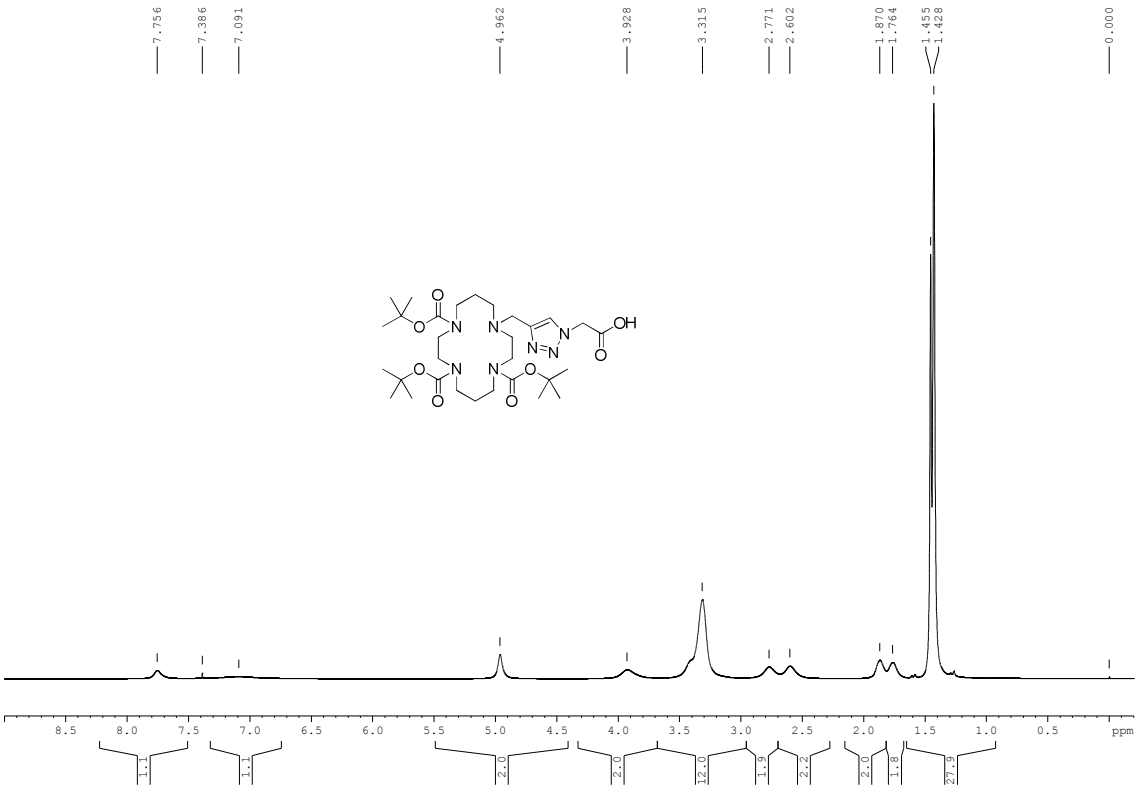


Figure S10. ^{1}H NMR spectrum (400 MHz) of **12** in $CDCl_3$.

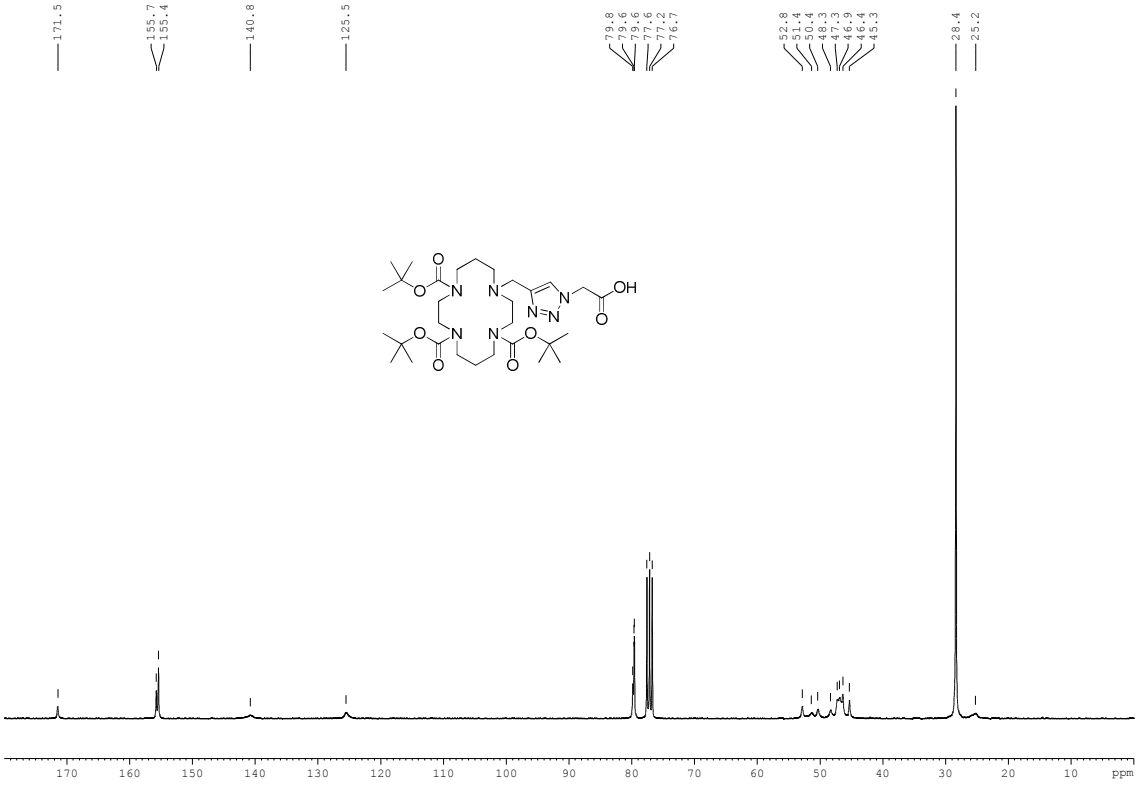


Figure S11. ^{13}C NMR spectrum (75 MHz) of **12** in $CDCl_3$.

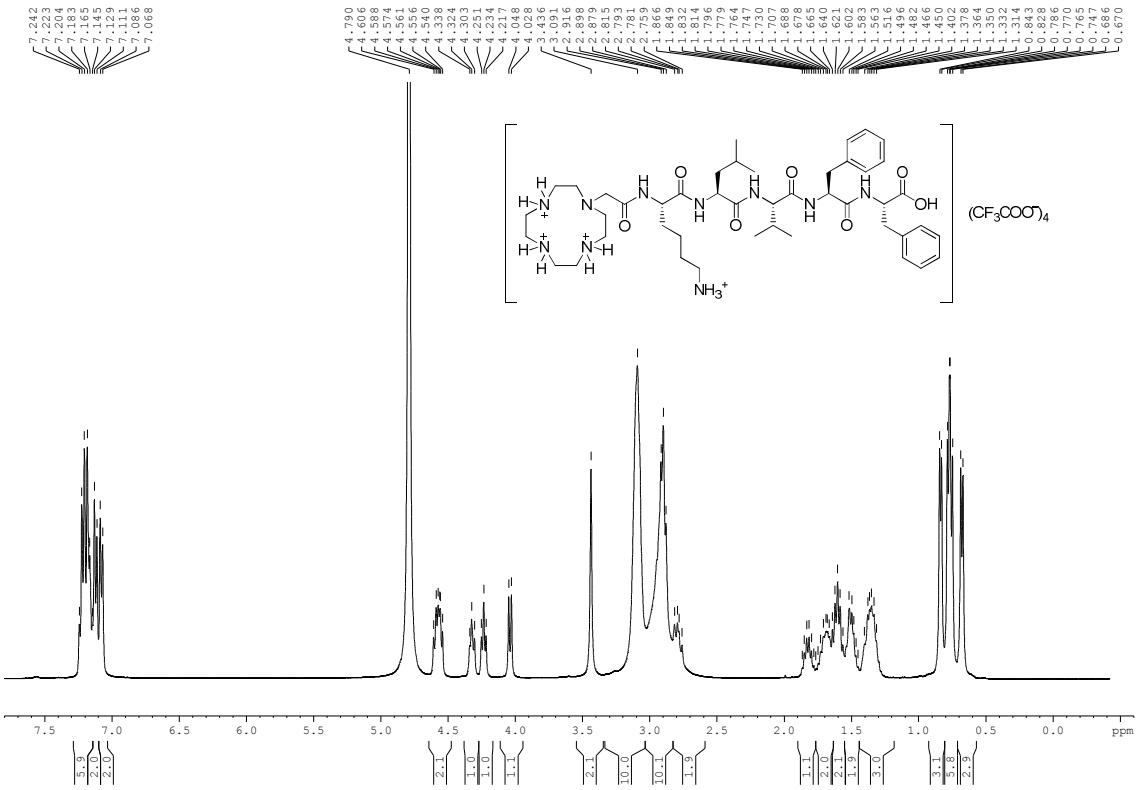


Figure S12. ^{1}H NMR spectrum (400 MHz) of **1** in D_2O.

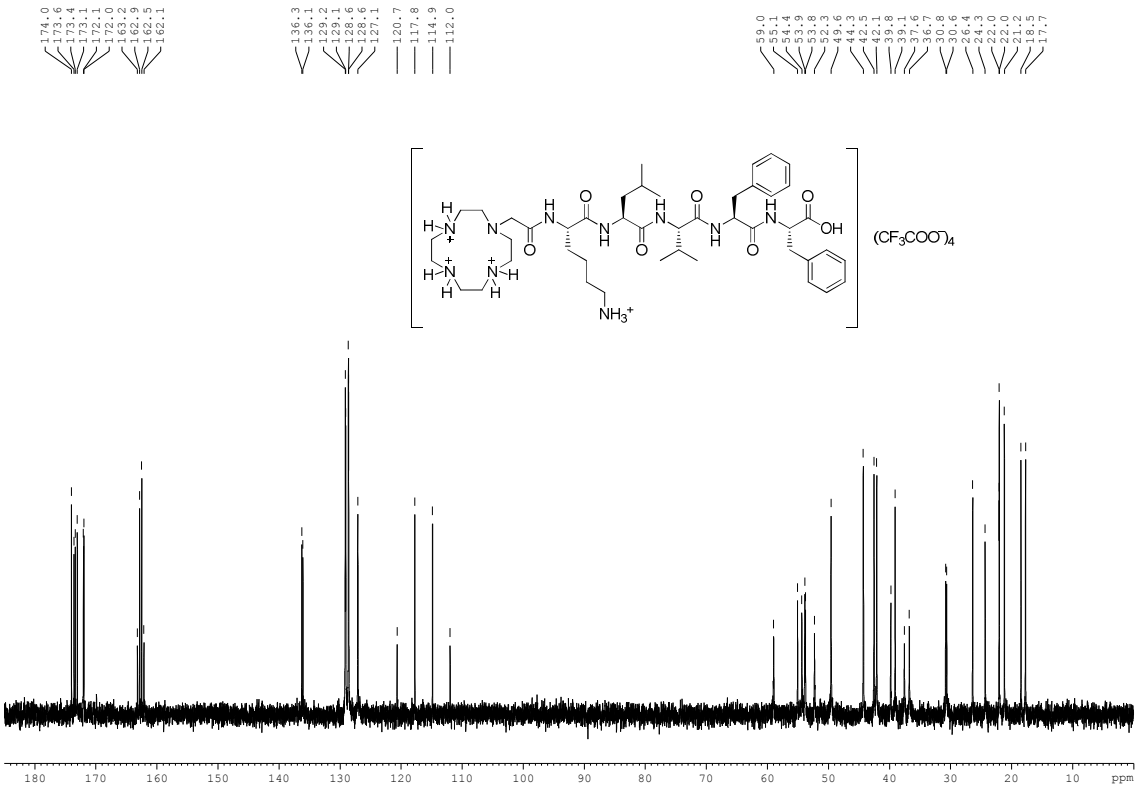


Figure S13. ^{13}C NMR spectrum (100 MHz) of **1** in D_2O.

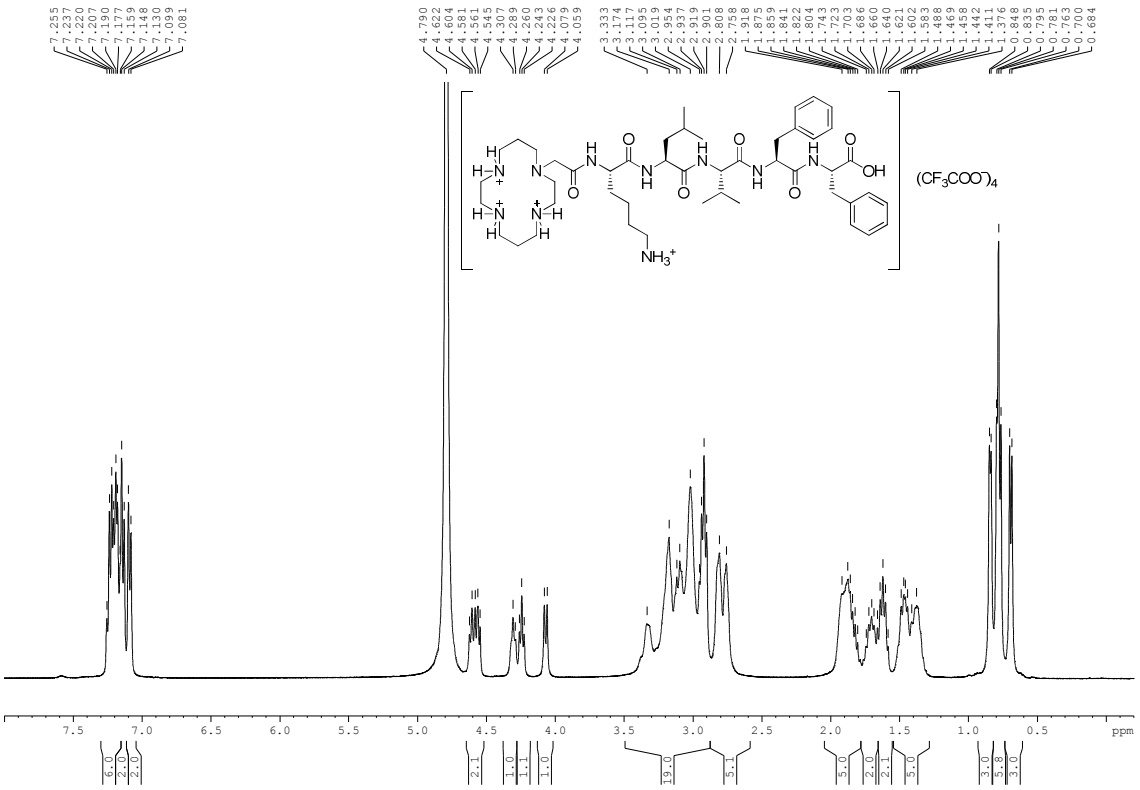


Figure S14. ^{1}H NMR spectrum (400 MHz) of **2** in D_2O.

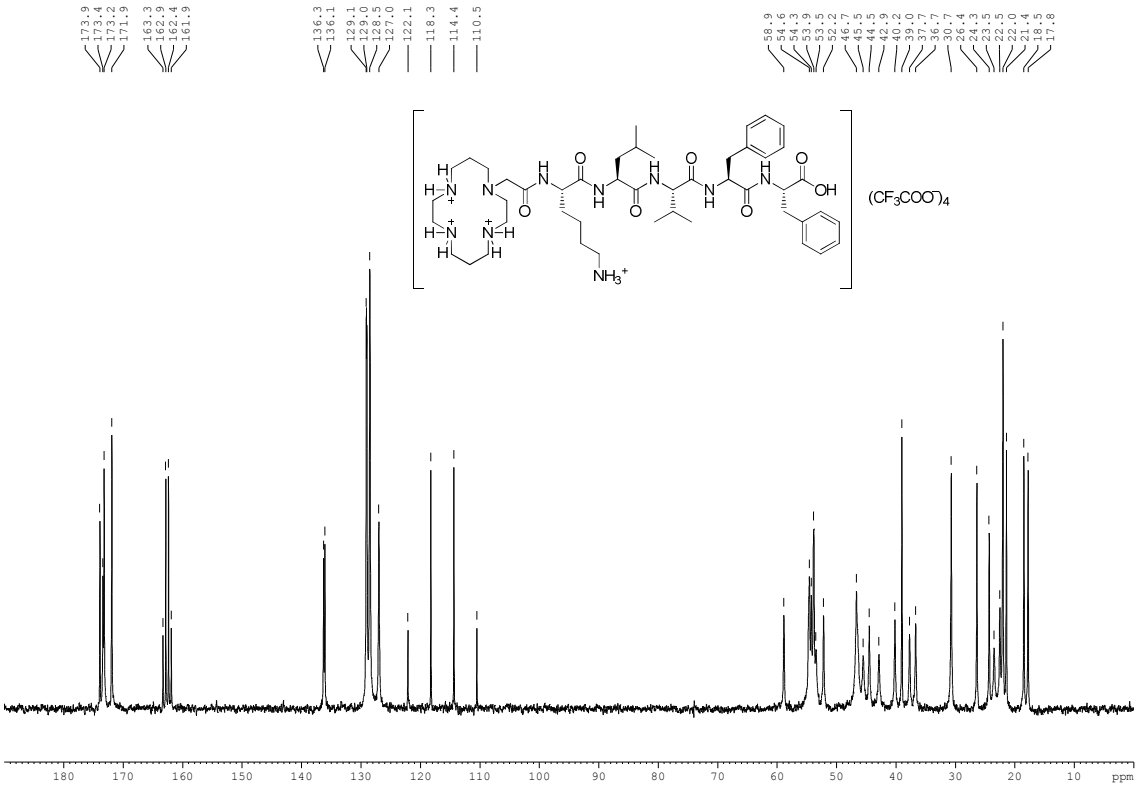


Figure S15. ^{13}C NMR spectrum (75 MHz) of **2** in D_2O.

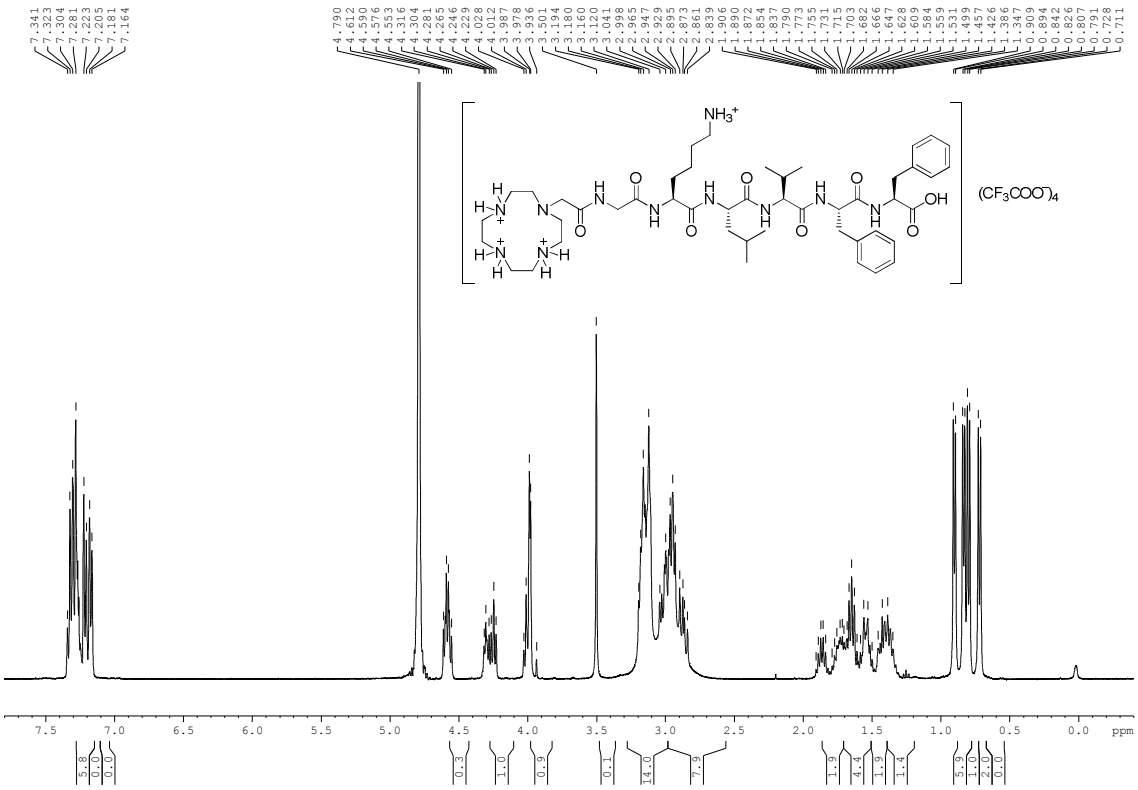


Figure S16. ^{1}H NMR spectrum (400 MHz) of **3** in D_2O.

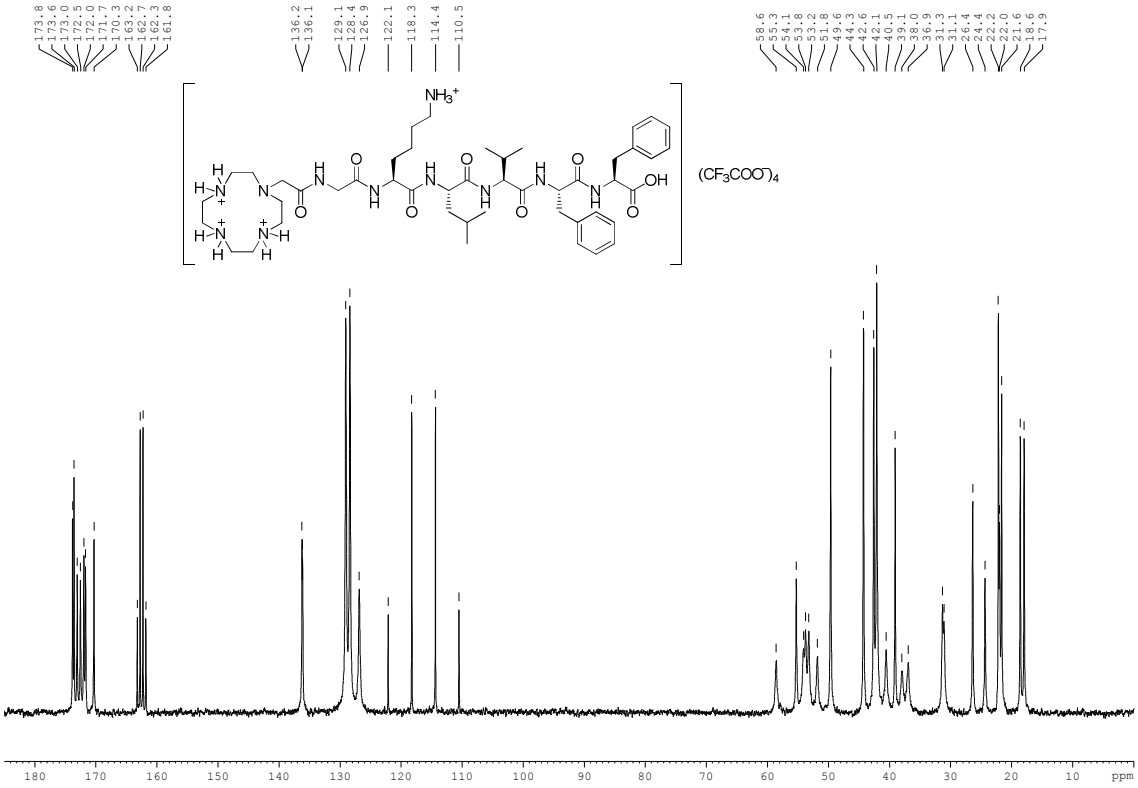


Figure S17. ^{13}C NMR spectrum (75 MHz) of **3** in D_2O.

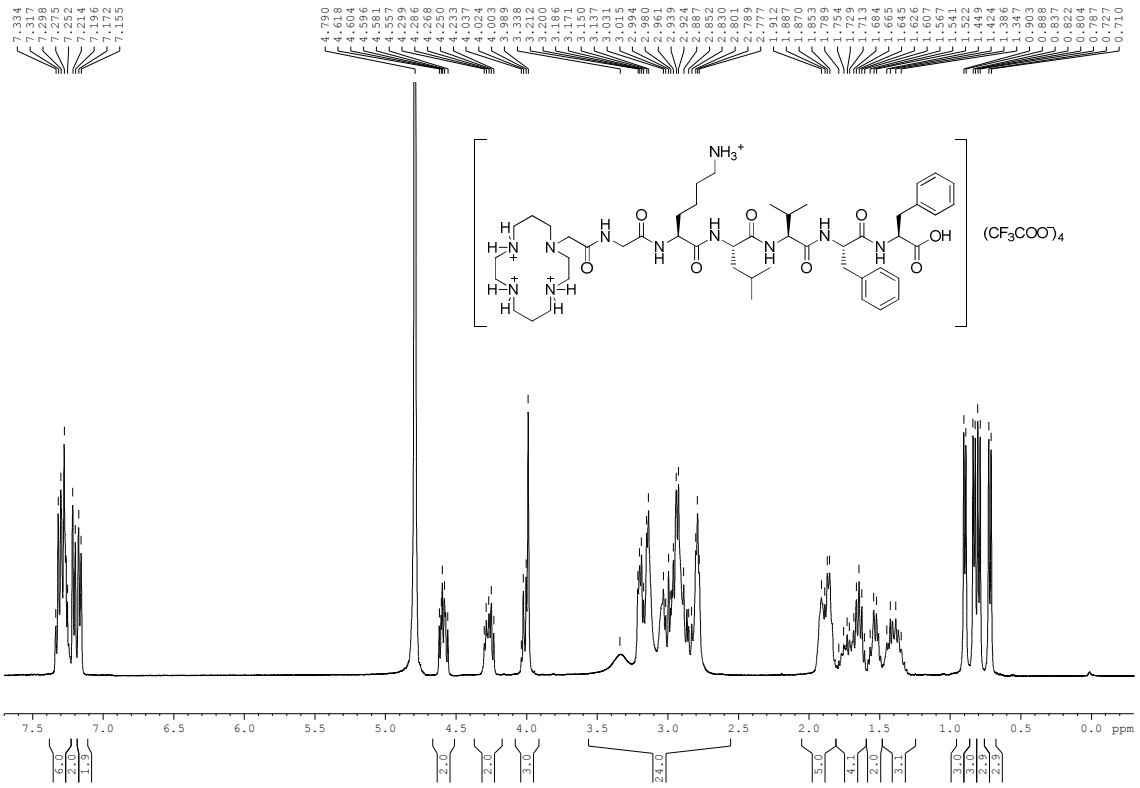


Figure S18. ^{1}H NMR spectrum (400 MHz) of **4** in D_2O.

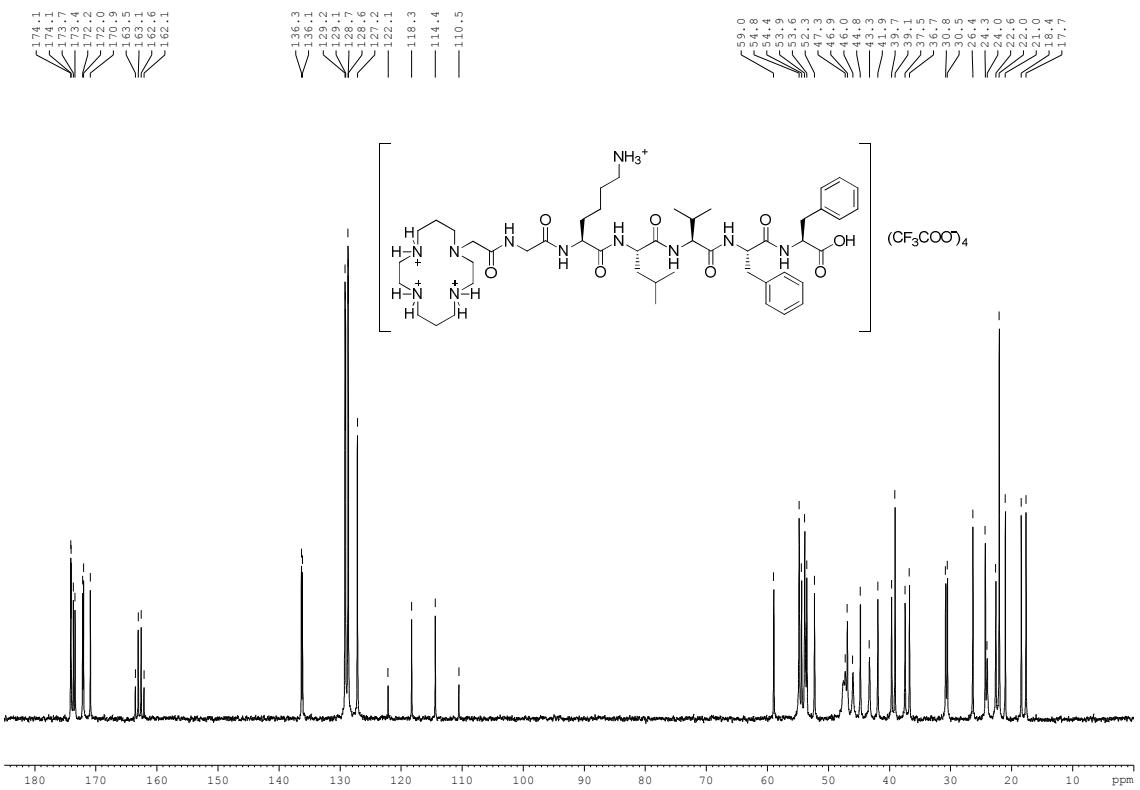


Figure S19. ^{13}C NMR spectrum (75 MHz) of **4** in D_2O.

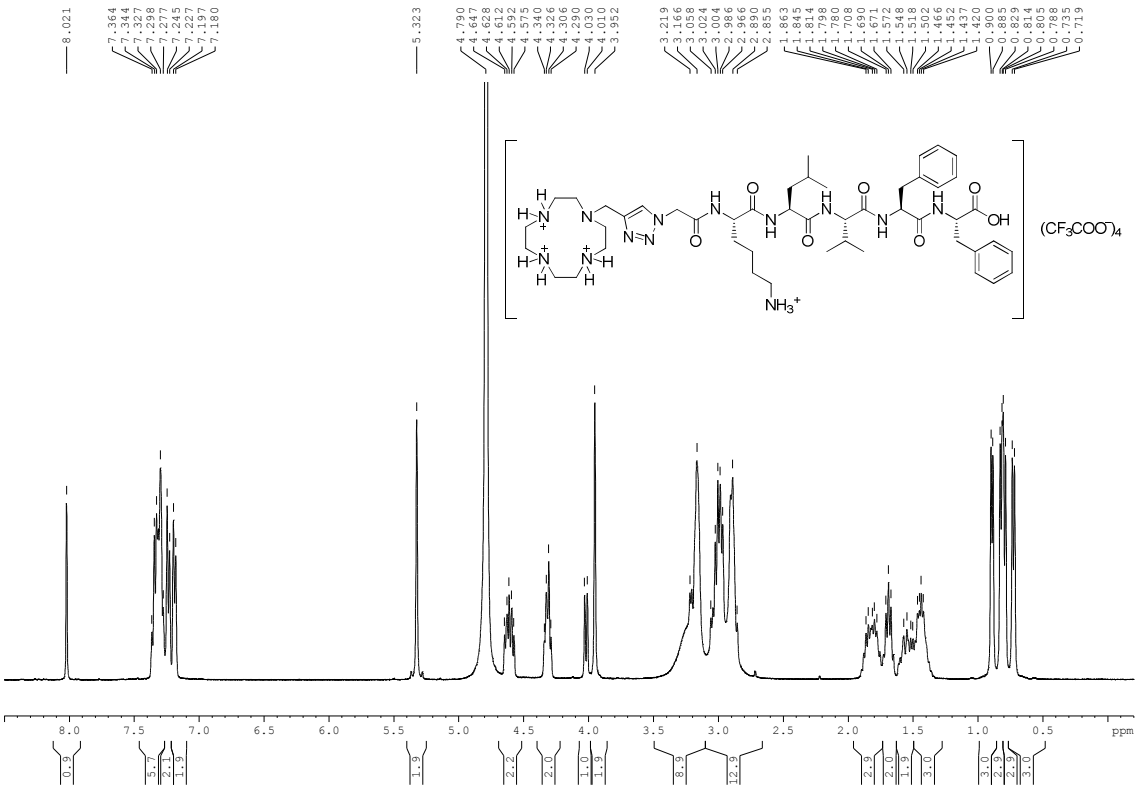


Figure S20. ^{1}H NMR spectrum (400 MHz) of **5** in D_2O.

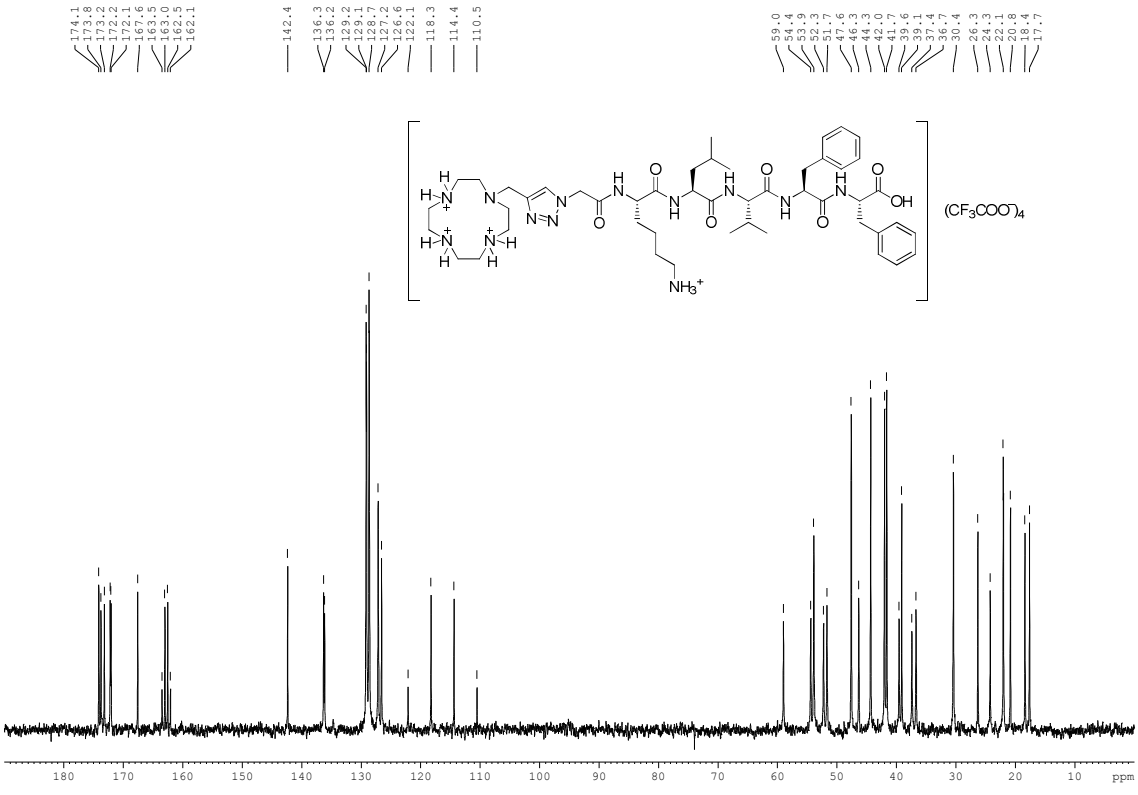


Figure S21. ^{13}C NMR spectrum (75 MHz) of **5** in D_2O.

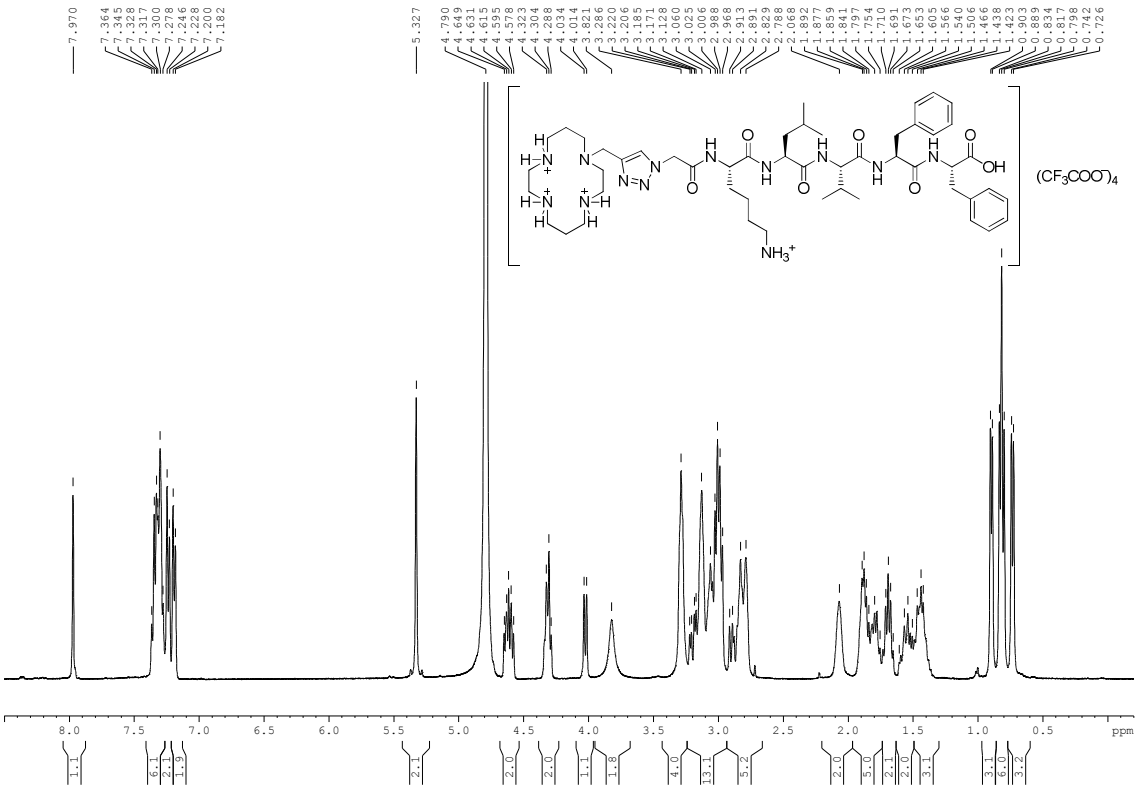


Figure S22. ^{1}H NMR spectrum (400 MHz) of **6** in D_2O.

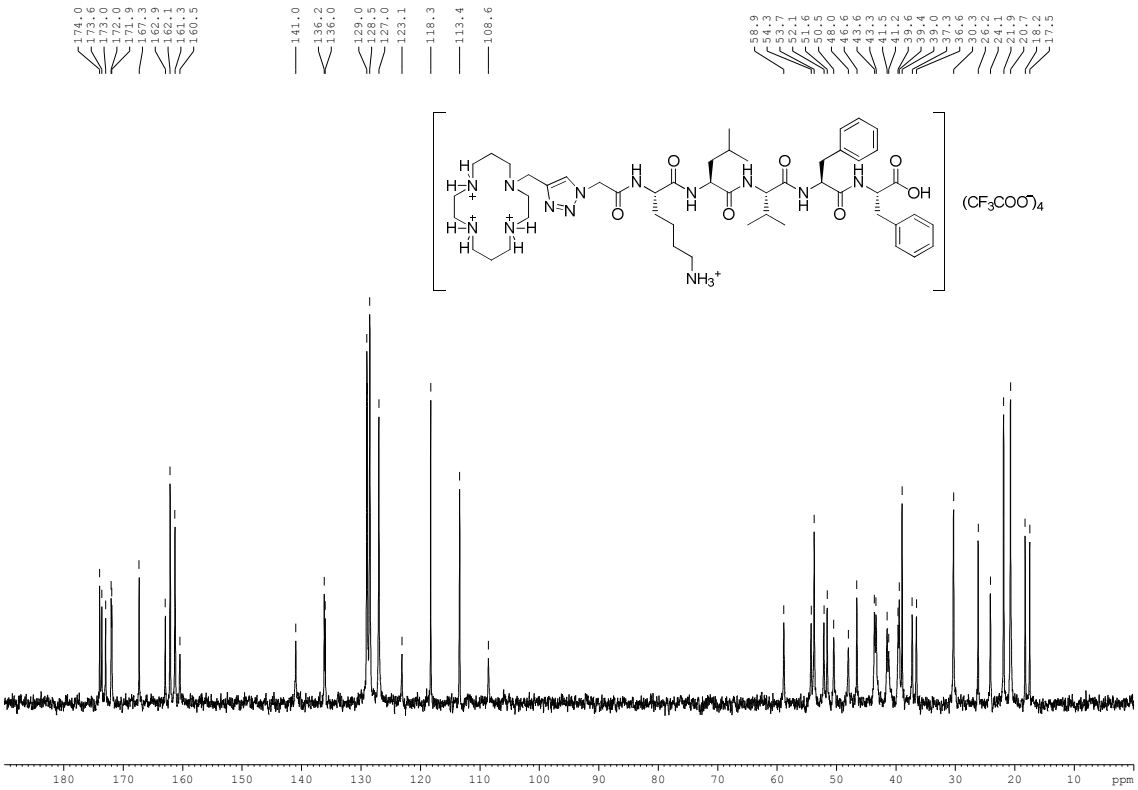


Figure S23. ^{13}C NMR spectrum (75 MHz) of **6** in D_2O.

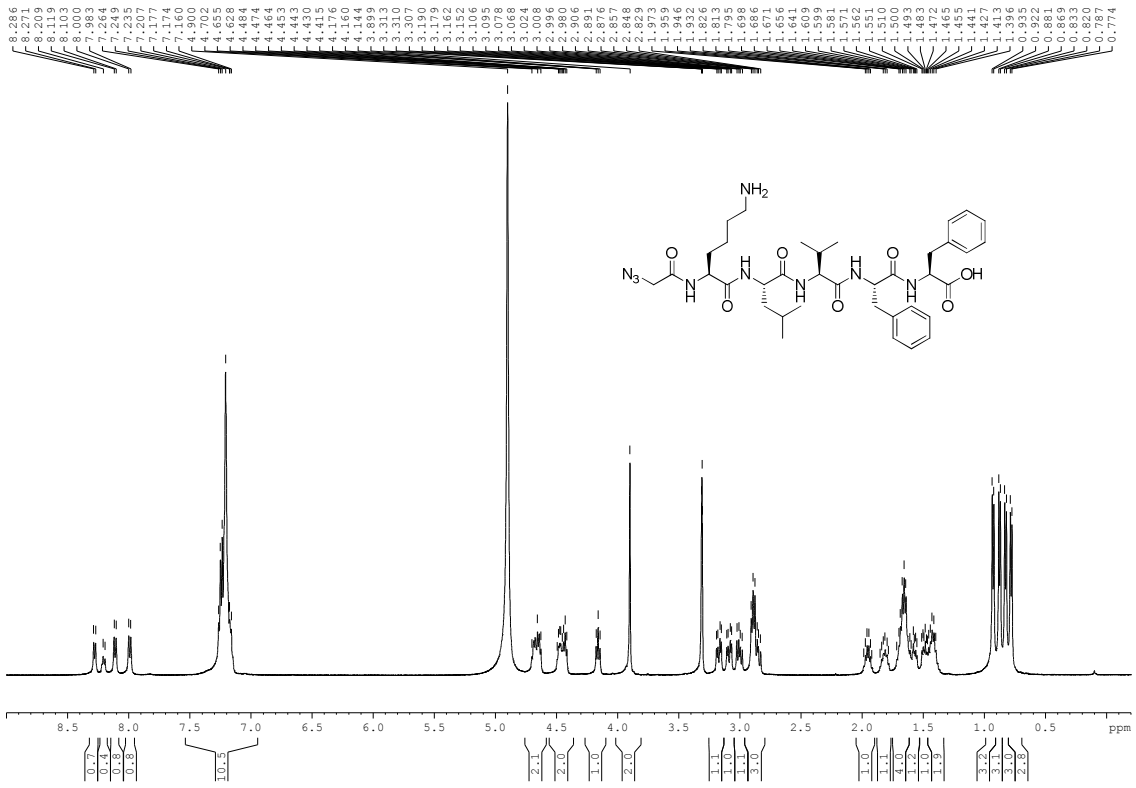


Figure S24. ^{1}H NMR spectrum (500 MHz) of **27** in CD_3OD.

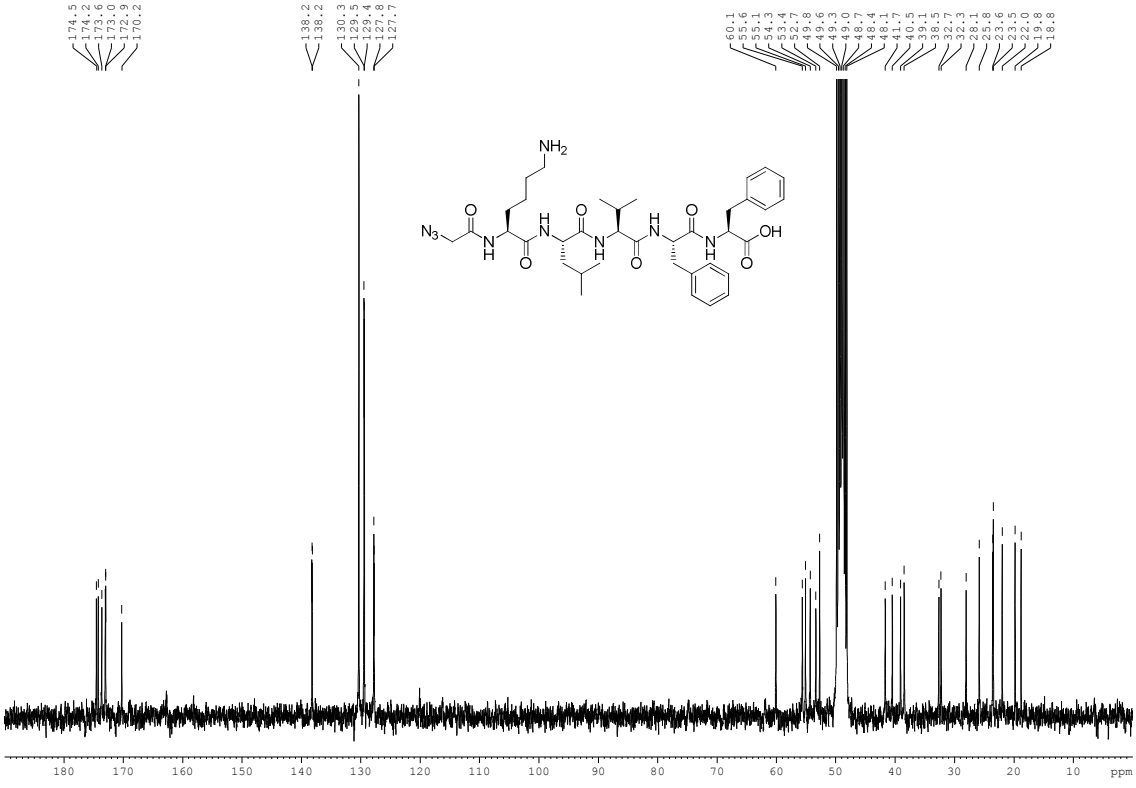


Figure S25. ^{13}C NMR spectrum (75 MHz) of **27** in CD_3OD.