Bis(thiosemicarbazones) as bifunctional chelators for the room temperature 64-copper labeling of peptides†

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A range of new carboxylate functionalised bis(thiosemicarbazone) ligands and their Cu(II) complexes have been prepared, fully characterised and radiolabeled in high yield with both ^{64}Cu and ^{99m}Tc. Conjugation to a bombesin derivative was achieved using standard solid phase synthetic methodologies and the ^{64}Cu-labeled conjugate was shown to have good tumour uptake in mice with xenografted PC-3 tumours.

Introduction

Target specific delivery of radionuclides for imaging of biological function has been of interest for several decades. The choice of radioisotope and bifunctional chelator are crucial for successful imaging and therapy, chelators should be easily attached to the peptide or protein and radiolabeling conditions must be biocompatible. ^{64}Cu ($t_{\frac{1}{2}}$ = 12.7 h, E_{av} = 278 keV) is both a β^+ and a β^- emitter allowing it to be used for both imaging and radiotherapy.[1,2] There are many reports of the use of ^{64}Cu-labeled macrocyclic chelators such as DOTA and TETA for the labeling of biomolecules.[1-3] However, nearly all require elevated temperatures over prolonged periods for efficient labeling. A chelating system with fast labeling kinetics at room temperature, at near neutral pH and giving high yields would provide advantages in the labeling of sensitive biomolecules unable to withstand elevated temperatures and extreme pH ranges.

N_2S_2-type bis(thiosemicarbazone) ligands form stable neutral and planar complexes with Cu(II) ions. This type of complex has been investigated extensively for the imaging of hypoxia in the form of copper(II)-diacetyl-bis(*N*-4-methylthiosemicarbazone), Cu[ATSM]. Radiolabeling occurs instantly at room temperature and near physiological pH.[4,5] This enables facile labeling post conjugation and avoids multi-step radiosynthesis. Bis(thiosemicarbazones) offer advantages for practical kit formulation when a copper isotope is the desired radionuclide and this would facilitate successful translation into routine clinical diagnosis and therapy.[6-8]

We have previously reported the synthesis of the bis(thiosemicarbazone)ligand H_2ATSR/A which can be functionalised *via* the exocyclic pendant amine substituent (Fig. 1).[5] However, solid phase peptide synthesis mostly proceeds in a C-terminus to N-terminus fashion, and some peptides and proteins require conjugation *via* their N-terminus or amine residues in order to preserve the biological function of the C-terminus.[9] We have also earlier described the synthesis of a bifunctional ^{64}Cu bis(thiosemicarbazone) with a pendant carboxylic acid functionality for conjugation to biologically active molecules. This particular ligand system was found to suffer from purification and solubility problems.[10]

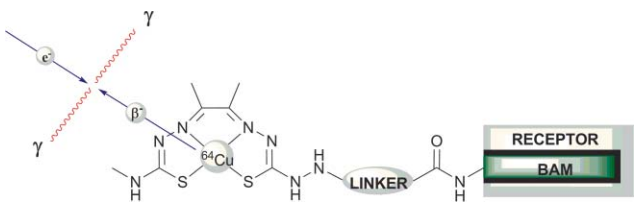

Fig. 1 Schematic diagram of a functionalised bis(thiosemicarbazone) conjugated to a biologically active molecule (BAM) for receptor targeted imaging.

In this paper, we report the synthesis of an entirely new series of bis(thiosemicarbazone) based copper(II) chelators bearing pendant COOH groups for the radiolabeling of peptides and proteins. We have opted for derivatisation of the ATSM core *via* the carboxyl functionality as this is compatible with traditional solid phase peptide synthesis methods. A number of chelating systems bearing aliphatic and aromatic spacers based on the H_2ATSR/A proligand have been synthesised, characterised and radiolabeled. Their suitability as bifunctional chelators and practical convenience have been assessed by subjecting them to standard solid phase peptide synthesis as well as solution phase coupling conditions commonly used for protein bioconjugation.

We chose a modified bombesin (BBS) sequence as model system as this provides substantially enhanced pharmacokinetics compared to the native form of the protein. Bombesin binds with high affinity to the gastrin releasing peptide receptors that are overexpressed by a variety of tumour cells such as prostate, breast, ovarian and small cell lung cancer.[11] Compatibility of the new

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ATSM derivatives with solution phase coupling conditions was demonstrated by reacting GlyGlyGly under aqueous conditions with the sulfo-NHS ester of the imine and amide bonded ligands.

Radiolabeling experiments with the thiosemicarbazone derived ligands and ^{99m}Tc were also successful. Initial evaluations of stability and serum binding show that the Tc species are stable in solution, show low protein binding and suggest that it may be possible to expand the scope of this ligand system to encompass SPECT as well as PET imaging. This provides a rare example of a ligand system that can be used for both technetium and copper in a radiopharmaceutical context.

Results and discussion

Ligand synthesis - imine conjugates

The H_2ATSR/A proligands **1** and **2** and the corresponding Zn complex **3** (Fig. 2) were synthesised as previously reported.[5]

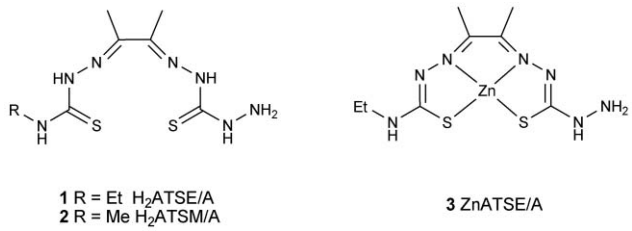


Fig. 2 Amine functionalised proligands and Zn(II) complex.

We were interested in routes to bis(thiosemicarbazone) ligands with reactive pendant carboxylic acid groups. We hoped that a simple ring-opening of succinic anhydride by ZnATSM/A **3** would provide the desired aliphatic carboxyl derivative **10a** (Fig. 3). However, we isolated a mixture of unidentified products over a range of temperatures from ambient to reflux in various solvent systems (THF, MeOH, DMF). Similarly, reaction of proligands of type **1** or **2** with succinic anhydride at room temperature resulted in an unidentifiable range of products. Reaction at higher temperatures resulted in a sparingly soluble precipitate. ^{1}H NMR and ESI-MS analysis of this compound suggested formation of a ring closed product **10b** (Fig. 3), indicated by one singlet resonance for both methylene protons at 2.8 ppm and a corresponding

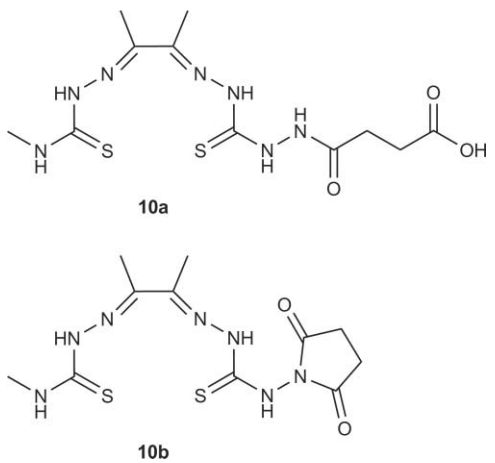


Fig. 3 Products of the reaction of H_2ATSM/A with succinic anhydride.

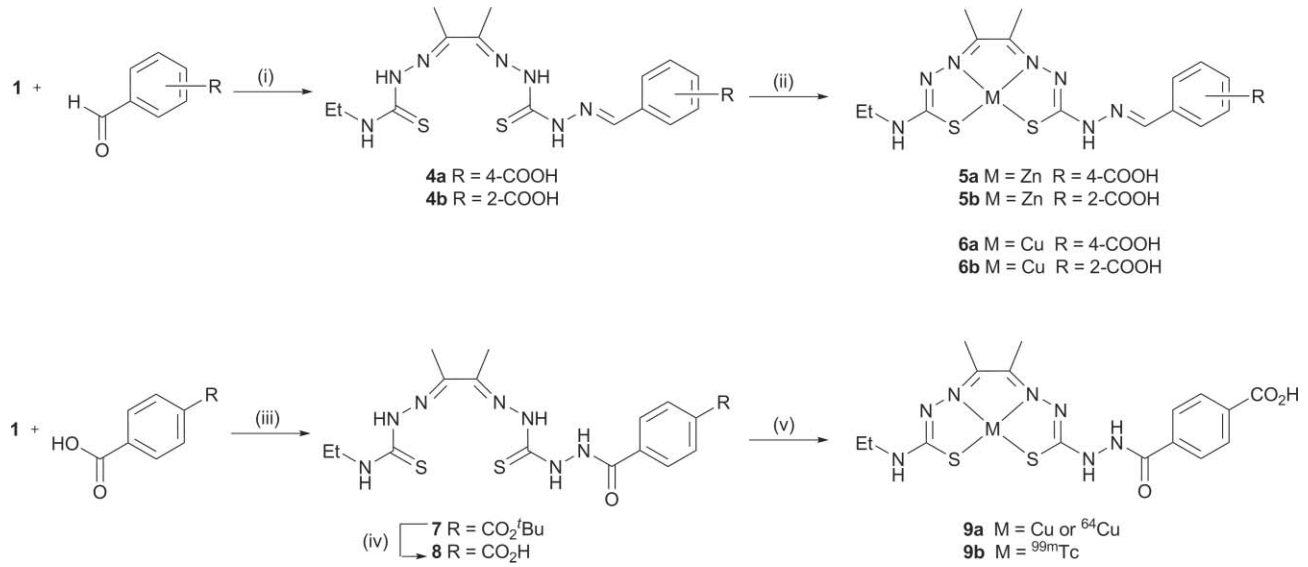
m/*z* of 342. The extremely low solubility prevented further characterisation.

Previous derivatives of **2** or **3** have been mainly based on aromatic imine conjugates, prepared by heating **2** under reflux in MeOH or EtOH with an aldehyde or ketone followed by Cu-transmetallation.[12,13] This prompted us to investigate phenyl-carboxylate imine derivatives which we anticipated would have improved solubility characteristics compared to the previously reported carboxylate derivatives.[10] Unfortunately, reaction of zinc complex **3** with 2- or 4-carboxybenzaldehyde proved problematic, giving multiple products based on ^{1}H NMR and HPLC analysis. Trial reactions with the corresponding methyl ester analogues, in contrast to the free acids, were found to proceed cleanly. Thus, the problems encountered may be due to the unprotected acid group binding to the free apical (fifth) coordination site on Zn either during reaction, isolation or analysis. We proceeded with the metal-free proligand **1** which was successfully reacted with 2- and 4-carboxybenzaldehyde at room temperature (Scheme 1). We were in fact able to show that the aromatic imine derivatives **4a/b** and **11a** (*vide supra*) mentioned within this paper, were formed in MeOH with the free proligands at room temperature or 40 °C overnight in yields of 70-85%. This completely avoided ligand self-cyclisation which was reported previously when carrying out such reactions at higher temperatures.[5] In contrast to other derivatives previously reported, complexation and transmetallation of **4a/b**, **5a/b** and **8** proceeded most efficiently with $ZnCl_2$ and $CuCl_2$ rather than the more commonly used metal acetates. Although the Zn complexes do not serve as synthetic precursors in this instance, we were still interested in their synthesis as we recently reported a solid supported method for purifying the ^{64}Cu-labeled complexes from their Zn precursors using controlled axial coordination.[8]

It was found throughout these experiments that derivatives of **1** proved easier to purify than those of **2** due to their slightly improved solubility in DMSO as well as protic solvents such as EtOH and MeOH at elevated temperatures.

Ligand synthesis - amide conjugates

As the aromatic imine derivatives could be susceptible to hydrolysis in the presence of water, we also investigated the synthesis of amide linked derivatives for improved stability. 1,4-benzenedicarboxylate motifs were used to avoid the possibility of cyclisation and the O^{t}Bu-monoprotected diacid was reacted with **1** or **2** in the presence of a range of coupling agents. Attempts to react **1** or **2** with the *N*-hydroxysuccinimide (NHS) activated esters of the monoprotected aromatic dicarboxylates resulted in incomplete reactions or multiple products. Coupling with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDCI) was also unsuccessful. Eventually we found that efficient coupling to **1** could be achieved with *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine. Compound **7** was synthesised cleanly in a yield of 96% using 4-(*tert*-butoxy-carbonyl) benzoic acid which was found to be more suitable than the mono-Me or Et esters which were partially hydrolysed during the coupling procedure and led to a mixture of products. Deprotection of **7** in neat TFA over 2.5 h proceeded efficiently to give **8**. It is noteworthy that exposure to TFA was found not to affect the bis(thiosemicarbazone) ligand system for periods of up to 4 h.



Scheme 1 Synthesis of imine and amide bonded functionalised bis(thiosemicarbazones) from the H_2ATSE/A proligand. (i) 1.2 eq aldehyde, MeOH, room temperature, 16 h (ii) 1.1 eq $Zn(OAc)_2{\cdot}2H_2O$ or $Cu(OAc)_2{\cdot}H_2O$, MeOH, rt, 30 min (iii) 1.1 eq BOP, 1.1 eq diisopropylethylamine, DMF, rt, 4 h (iv) trifluoroacetic acid (TFA), rt. 2.5 h (v) $Cu(OAc)_2{\cdot}H_2O$ (for radiolabeling conditions for **9a** and **9b** *vide supra*).

Amino acid conjugation and extended linker synthesis

To assess the aqueous phase coupling behaviour of our ligands with peptides and proteins we wanted to couple **4a**, **4b** and **8** (Scheme 1) to glycine and triglycine as model amino acid and peptide sequence mimic respectively. The activated NHS esters of **4a/b** and **8** were readily formed using EDCI in DMF. However these intermediates did not react readily with glycine or triglycine sequences and were recovered unchanged. This may be due to the general lack of reactivity of aromatic carboxylates compared to their aliphatic counterparts compounded by a stabilising conjugation with the ATSE ligand system. Interestingly, coupling of the pendant aromatic COOH group of **8** to glycine(*tert*-butylester) was acomplished as previously with BOP and *N*,*N*-diisopropylethylamine to give the corresponding conjugate **11b** in 98% yield (Fig. 4). Deprotection conditions analogous to those for **7** afforded the extended linker **11c** (Fig. 4). In the case of **4a**, the extended linker was obtained by condensation of **1** with the appropriate aldehyde methyl 2-(4-formylbenzamido)acetate.[14] Copper complexation to give **12a** and **12c** was quantitative for all the ATSE proligands with extended linkers and occurred rapidly at room temperature in MeOH.

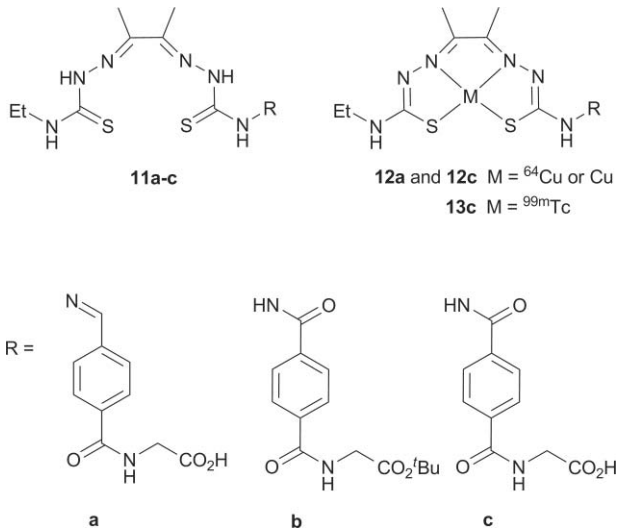


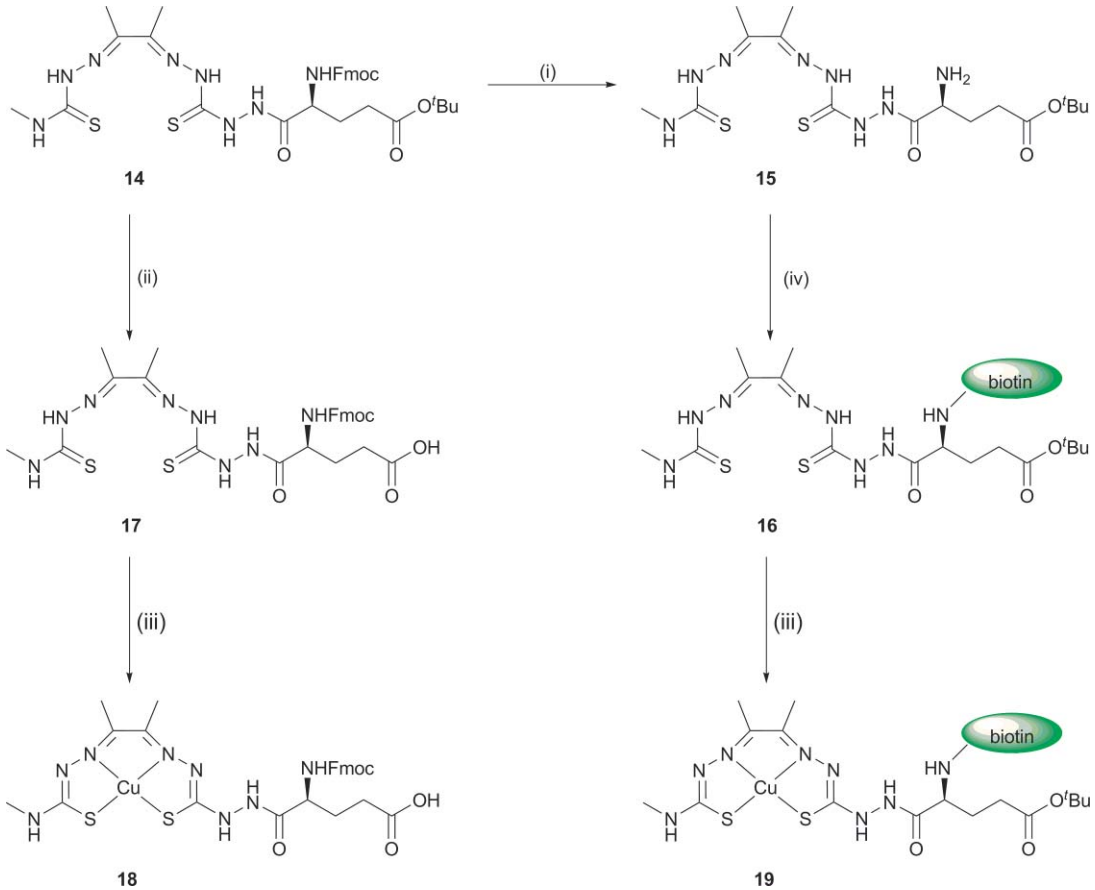
Fig. 4 Extended linkers.

Our extended linkers **11a** and **11c** were successfully converted to their water soluble sulfo-NHS ester analogues using EDCI and sulfo-NHS in DMSO. Coupling was achieved within 15 min as confirmed by ESI-MS, and the sulfo-NHS esters of ligands **11a** and **11c** were reacted with triglycine in DMSO or in buffered aqueous conditions. Reactions were monitored by ESI-MS and showed that after one hour the desired conjugate could already be detected and after 12 h only product and residual **11a** (when conducted in H_2O) were detected but no starting active ester as in the case of the non-extended linkers (see Supplementary Information for structures). These preliminary experiments demonstrated that our extended linker derivatives are in principle suitable for conjugation to pendant amino groups on larger biomolecules such as proteins under aqueous conditions using a frequently employed EDC/sulfo-NHS approach.[15]

Bifunctional derivatisation–conjugation with glutamic acid

We were also interested in preparing a bifunctional chelator that, in addition to being capable of conjugation to a targeting biomolecule, could potentially accommodate another orthogonal biomarker.

In order to further enhance the solubility we wanted to provide ligands bearing aliphatic spacers. Thus, we chose to couple our proligands **1** and **2** to glutamic acid with orthogonally-protected NH_2 and CO_2H groups. Attempts to couple **1** or **2** *via* a NHS-activated glutamic acid derivative led to incomplete reactions or non-purifiable products. The synthesis of **14** (Scheme 2) was realised by coupling of **1** with FmocGlu(O^{t}Bu)OH in an analogous fashion to the conditions used for ligand **7**. Selective deprotection



Scheme 2 Synthesis, selective deprotection and bioconjugation of aliphatic linked bis(thiosemicarbazone) bifunctional chelators. **14** and **16** were prepared under the same conditions as for **7** using BOP. (i) 20% piperidine, DMF, 45 min then H_2O, then Et_2O. (ii) TFA, 3 h, rt then Et_2O. (iii) $Cu(OAc)_2 \cdot H_2O$, MeOH, rt, 30 min. (iv) Biotin, BOP, DIPEA, DMF, 4 h.

to give the free amine or free acid was easily achieved with standard Fmoc/^{t}Bu deprotection methods using 20% piperidine in DMF to give **15** and neat TFA over 3 h to give **17** followed by Cu-metallation to give **18**. As proof-of-principle, **15** was conjugated to the commonly used biomarker biotin. Subsequent Cu-complexation to form **19** proceeded with a 96% yield.

The successful, selective deprotection, conjugation and facile complexation with copper suggest that **15** and **17** may be suitable for simultaneous attachment of further pretargeting groups as well as offering the possibility of incorporating a radiotracer at any point within a peptide sequence.

Radiolabeling

^{64}Cu. **8**, **11a**, and **11c** were radiolabeled by direct reaction of $[^{64}Cu](OAc)_2$ with the corresponding pro-ligand as reported for previous systems.[5] All complexes were labeled with radiochemical yields >99% and high radiochemical purities (radio-TLC, >95%). As anticipated for bis(thiosemicarbazone) systems, all labeling occurred at room temperature virtually instantaneously. Radio-HPLC analysis within a few minutes of labeling indicated that no peaks resulting from unbound residual $^{64}Cu(OAc)_2$ were present.

^{99m}Tc. We were also interested to see whether our bis(thiosemicarbazone) ligand systems could also be employed for other radionuclides. Kethoxal-bis(thiosemicarbazone) (KTS) is reported to have been radiolabeled with $^{99m}TcO_4^-$ to form an uncharged ^{99m}Tc(IV) complex, ^{99m}Tc-KTS, which it was suggested to be stable in aqueous media.[16] The labeling of ATSM and simple alkylated analogues with ^{99m}Tc has also been reported.[17] Interestingly the ATSM complex was shown to be hypoxic selective in isolated ischaemic heart tissue.[17] However, in neither paper was the structure of the Tc complex established. Ligands **8** and **11c** were radiolabeled according to a modified procedure by complexation with ^{99m}Tc using $SnCl_2$ as a reducing agent in sodium carbonate buffer at 95 °C.[18] Pertechnetate and other hydrophilic impurities were removed using a Sep-pak™ C18 cartridge[19] to give the products **9b** and **13c** in non-optimised radiochemical yields of 30–40% (Table 1). The Sep-pak™ method potentially removes some of the target ^{99m}Tc-complexes and hence the actual radiolabeling efficiency might be higher.[19] After purification, the radiochemical purities of both complexes were >95% as determined by radio-TLC using MeOH:10% NH_4Ac (aq) (1 : 1) as solvent.[16] The Tc-colloid remains at the starting point, while the radiotraces of both complexes moved with the solvent front (see Supplementary Information†).

This suggests that both derivatives labeled successfully, but further optimisation is needed to increase the yield. Experiments are currently underway to determine the structures of the Tc complexes using the long-lived ^{99}Tc isotope and the biological

Table 1 Radiolabeling yields and a comparison of serum binding and log *P* values for ^{99m}Tc and ^{64}Cu labeled carboxylate derivatives

Compound	RCY	Serum Binding (120 min)	Log P
CuATSM	99%	20%	1.5
9a	>99%	20%	1.48
9b	60%	26%	0.28
12c	>99%	19%	0.88
13c	50%	32%	–0.41

behaviour of the ^{99m}Tc labeled bis(thiosemicarbazone) bifunctional chelators *in vitro* and *in vivo*.

Serum binding studies of ^{64}Cu and ^{99m}Tc labeled derivatives

The serum stability and protein binding of our new derivatives were determined for comparison with the parent compound Cu[ATSM]. The ^{64}Cu and ^{99m}Tc-labeled analogues **9a**, **9b**, **12c** and **13c** and were incubated in mouse serum for 120 min and samples were taken at different time points. After 5 min, approximately 20% of the activity was bound to the proteins for the ^{64}Cu analogues. This is comparable to what has been observed for CuATSM and other bis(thiosemicarbazones) previously which have been tested *in vivo*.[4,12]

The ^{99m}Tc-labeled analogues had a higher degree of binding to serum proteins at around 30% but the total activity bound remained constant over time, suggesting little or no decomposition over the time course by analogy with the Cu-labeled ATSM derivatives. The slightly lower log *P* values of the ^{99m}Tc-labeled complexes are consistent with the more hydrophilic nature of a proposed Tc oxo-core and the Tc complex may be charged

SPPS synthesis of chelator-BBS conjugates

The ability to survive standard solid phase peptide synthesis conditions is important if the chelator is to be incorporated as a tag in a peptide sequence. The stable amide-bonded chelators **8** and **11c** were deemed most suitable for SPPS.

BBS sequences have been extensively labeled with various radionuclides such as ^{18}F, ^{64}Cu, ^{67}Ga, ^{99m}Tc and ^{177}Lu to provide tumour specific tracers for receptor imaging.[20-23] The sequence chosen was the minimum active sequence BBS(7–14) modified to achieve enhanced pharmacokinetics. It bears a β^{3}hAspβAlaβAla spacer between peptide and chelator as well as Leu^{13} and Met^{14} replacement by Cha and Nle respectively.[24,25] A higher tumour-to-background ratio and increased stability were also reported. The chosen sequence has been previously labeled with ^{99m}Tc using an (N$^{\alpha}$His)Ac chelator for the radiometal thus a platform for comparison of future *in vitro* and *in vivo* evaluations would be available.[26,27] The protected peptide was synthesised manually on a Rink Amide Resin using the Fmoc-strategy previously described.[25]

Table 2 Chemical characterisation of bis(thiosemicarbazone) bombesin conjugates

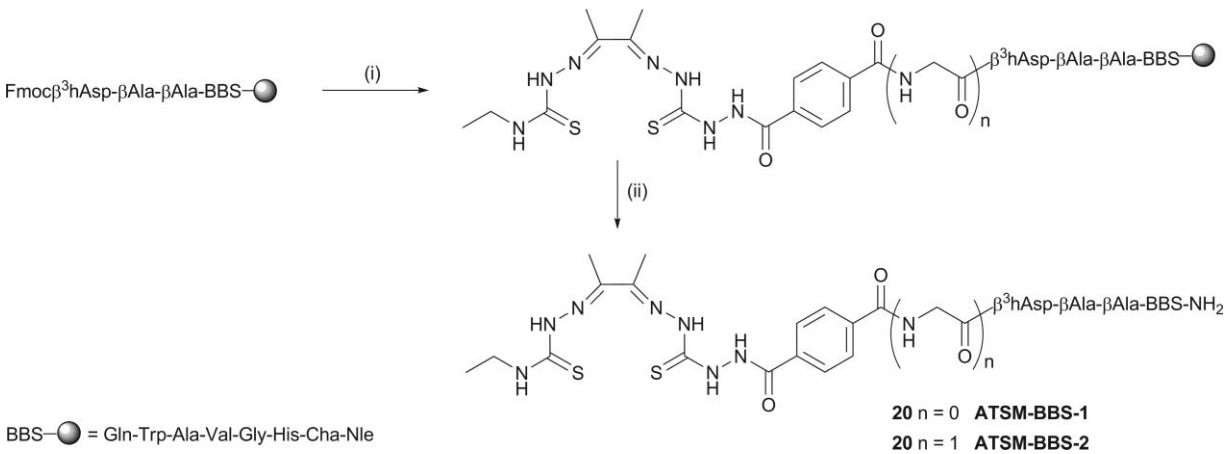
Compd	*m*/*z* found(calcd)	% yield	purity	HPLC t_R/min
ATSM-BBS-1	1638.6 (1637.8)	53.4%	94%	14.1 min
ATSM-BBS-2	1694.7 (1694.8)	19.4%	95%	14.0 min

Again, coupling of chelator **8** with standard DIC coupling conditions posed difficulties but coupling to the resin-bound BBS sequence using BOP proved successful (Scheme 3). Chelator **11c** coupled easily using standard DIC coupling conditions as anticipated due to the introduction of the Gly residue. Both conjugates of **8** and **11c** were then cleaved from the resin by treatment with TFA for 3.5 h and lyophilised to afford **20** (ATSM-BBS-1) and **21** (ATSM-BBS-2) respectively. Preparative HPLC and lyophilisation of the conjugates resulted in chemical purities that compared well with previously synthesised BBS-chelate systems (Table 2).[28]

EPR studies

Before proceeding to radiolabel our ATSM-BBS conjugates, we wished to see whether metal binding at ambient temperature would still result in specific Cu complexation at the chelator site rather than unspecific binding to the peptide sequence. We titrated both **8** and **ATSM-BBS-1** with 1 eq of $Cu(OAc)_2 \cdot H_2O$ at room temperature.

The EPR spectra of both **9** and **CuATSM-BBS-1** (Fig. 5) were indicative of a single copper(II) species being formed, with no evidence for other binding modes observed. The copper is d^9



Scheme 3 Solid Phase Synthesis of the bisthiosemicarbazone-BBS conjugates: Reagents and Conditions: (i) 1 ml 25% 4-methylpiperidine in DMF, 2 ×10 min, rt then ATSM-BBS-1: 2 eq BOP, DIPEA, DMF, rt, 8 h; ATSM-BBS-2: 2 eq DIC, HOBt, 3 h, rt (ii) thioanisole (TA)/ethanedithiol (EDT) 7 : 3 in TFA, 3.5 h.

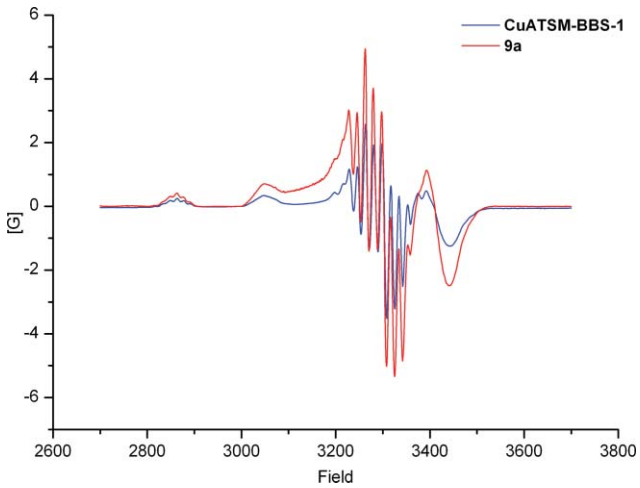


Fig. 5 X-band EPR spectra of **9a** (red) and Cu-labeled **20** (blue) in anhydrous DMF/ethylene glycol 4 : 1 at 60 K.

square planar and the EPR spectra at X-band bear close similarity to other Cu(II)ATSM derivatives previously studied.[29-34] The $g_{\|}$ values of 2.1299 were less than 2.3, which indicates covalent character for the metal–ligand bonds.[34] This was found to be the case for Cu(II)ATSM and was supported by multiple DFT calculations.[32] NPA (natural population analysis) depictions of the spin densities of Cu(II)ATSM indicated that a high degree of covalency was present in the coordination sphere.[32] As expected, the g_z and ACu_z are the largest components to the hyperfines as the unpaired electron is held in the dx^2-y^2 orbital and the largest component is in the g_z direction due to the cancelling out symmetry of the orbital in the xy plane. The superhyperfine couplings were not well resolved, as the molecules were not completely symmetrical, making the coordinating nitrogens inequivalent. This is in contrast to another derivative Cu(atsm/apyrene),[33] in which the two nitrogens were reported to be equivalent. However, since the reported spectrum was measured at room temperature, this is likely to be a result of molecular tumbling averaging out the anisotropy of the g-tensors.

EPR analysis thus confirmed site specific labeling for our isolated CuATSM-BBS-1 conjugate and its Cu-labeled congener.

$^{64/67}$Cu-radiolabeling of ATSM-BBS conjugates

Labeling was carried out in ammonium acetate buffer at pH 5.5 and as anticipated proceeded cleanly at room temperature. Both derivatives labeled in high radiochemical yield, the yield for $^{64/67}$CuATSM-BBS-2‡ however was slightly higher than that for $^{64/67}$CuATSM-BBS-1. The radiochemical yields for $^{64/67}$CuATSM-BBS-1 and $^{64/67}$CuATSM-BBS-2 were 91% and 85% respectively. Labelings done at 75 °C resulted in yields > 90-95%. HPLC analysis after 24 h showed that the complexes were stable in solution. Log *P* values were 0.42 ± 0.04 and 0.39 ± 0.01 for $^{64/67}$CuATSM-BBS-1 and $^{64/67}$CuATSM-BBS-2 respectively.

‡ The radiocopper used for the protein labeling and *in vitro* and *in vivo* experiments contained small amounts of ^{67}Cu. Pure ^{64}Cu was used for all other labeling work.

Receptor binding and internalisation studies

The binding affinity of the ligand-peptide conjugate is an important prerequisite parameter for high *in vivo* tumour uptake. We performed binding studies with the unlabeled ATSM-BBS-1 and ATSM-BBS-2 on human prostate adenocarcinoma PC-3 cells which express a high level of GRPR. The affinity of both analogues was similar (see Fig. 6). The IC_{50} values for **20** (ATSM-BBS-1) and **21** (ATSM-BBS-2) were 2.9 nM and 3.8 nM, respectively, which is comparable to that of natural bombesin (1.9 nM). Furthermore these values compared well to the analogous (N^{α}His)chelator containing sequence previously used for ^{99m}Tc (5.1 nM).[26,27] The bis(thiosemicarbazone) chelating system thus does not seem to have any adverse effect on the receptor affinity of the peptide.

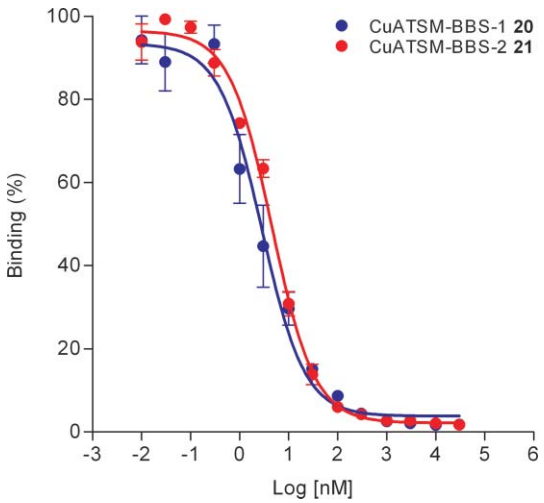


Fig. 6 Displacement curves of ATSM-BBS-1 and ATSM-BBS-2 in PC-3 cells.

Receptor internalisation was somewhat slower for the $^{64/67}$CuATSM-BBS conjugates than for the comparable ^{99m}Tc containing systems. The non-specific binding was 40% for $^{64/67}$Cu-ATSM-BBS-1 and 20-30% for Cu-ATSM-BBS-2 and is higher than for BBS labeled with ^{99m}Tc(N^{α}His).

Overall, CuATSM-BBS-2 showed much better internalisation than CuATSM-BBS-1 (see Supporting Information). Pleasingly, the internalisation of CuATSM-BBS-2 at 40% is comparable to the ^{99m}Tc radiolabeled analogues which gave values of 30-45% (15% if there are two negative charges involved in the spacer between peptide and chelator).

Preliminary *In vivo* biodistribution

Preliminary biodistribution data was obtained using $^{64/67}$CuATSM-BBS-2 in nude mice with PC-3 tumour xenografts. Two groups of 3 mice were injected intravenously with 100 KBq of the $^{64/67}$Cu complex and sacrificed 1 and 24 h post injection (p.i.). Fig. 7 shows the biodistribution of the complex at the two time intervals as % injected dose/gm (%ID/gm) averaged over 3 mice.

At 1 h p.i. there is good uptake in the tumour with a tumour/muscle ratio of 9.6 : 1. Significantly this value was reduced by about 52% in a third group of animals pretreated with natural bombesin at a dose of 0.1 mg per mouse. This reduction

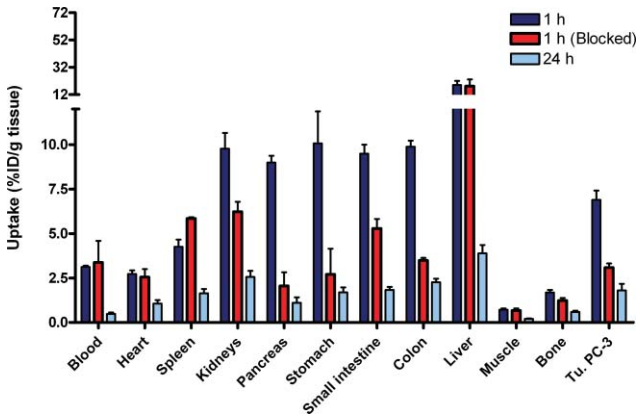


Fig. 7 Biodistribution of $^{64/67}$Cu-ATSM-BBS-2 in nude mice with PC-3 tumour xenografts (100 KBq/mouse i.v.).

is comparable with values obtained in blocking studies with bombesin conjugates using other radioisotopes. Lung uptake was high at early p.i. times, probably due to the low solubility of CuATSM-BBS-2 in aqueous biological media but it was rapidly cleared. Liver uptake is high, which is in accordance with the high lipophilicity, and the possibility that some copper is sequestered from the ATSM ligand by the liver. The relatively high uptake in both pancreas and gastrointestinal tract is consistent with the high expression of bombesin receptors in these tissues. Both these values were substantially reduced in the blocking experiment, confirming the specificity of the *in vivo* uptake. The nature of the radiometal chelator, the linking group and the structural variant of bombesin used all have a impact on the biodistribution of the labelled BBS peptide.

However the tumour uptake here appears higher than with any other copper chelators that have been used with the same bombesin structural motif.

Further experiments are in progress with conjugates designed to be more hydrophilic to assess the impact on both cell internalisation and biodistribution. The results of this work and a more detailed comparison of the data with other radioisotopes will be published at a later stage.

Conclusion

We have synthesised and characterised a new series of bis(thiosemicarbazone) bifunctional chelators, incorporating both rigid aromatic and aliphatic linkers to carboxylate groups. We have successfully conjugated them using both solid and solution phase techniques. Preliminary biological data for the new bombesin bioconjugates described above demonstrate their suitability for use in biological systems. Room temperature radiolabeling and full EPR characterisation demonstrate that rapid labeling and site specificity are maintained when conjugated to a biologically active molecule. Initial *in vitro* and *in vivo* evaluations of these first generation bis(thiosemicarbazone) functionalised chelators show that they are in principle suitable for protein targeted PET imaging. Moreover the ^{99m}Tc-labeling experiments show that these new carboxylate derivatised ATSM derivatives are also promising for the SPECT labeling of proteins. These new derivatives thus offer a viable and flexible route for labeling of sensitive biomolecules for targeted imaging. Experiments are ongoing to optimise the *in vivo* behaviour of the conjugates by further modifications of the ATSE ligand system.

Experimental

General experimental techniques

Chemicals were obtained from Acros, Aldrich, Alfa Aesar, Apollo Scientific or Bachem unless otherwise stated. All solvents used were HPLC grade. TLC, where applicable, was performed on pre-coated aluminium-backed plates (Merck Kieselgel) and spots were made visible by quenching of UV fluorescence (λ = 254 nm) and/or by staining with potassium permanganate. Flash chromatography was performed according to the method by Still, using silica gel (0.040-0.063 mm; Merck) and air pressure. NMR spectra were recorded on a Varian Mercury VX300 or a Bruker AVANCE AVC500 at frequencies of 300 MHz and 500 MHz for ^{1}H and 75.5 MHz and 126 MHz for ^{13}C respectively, using residual solvent peaks as internal reference.[35] ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC. Nominal mass spectra (m/z) were recorded on a Fisons Platform II, under the conditions of positive or negative electrospray ionization (ESI-MS). High resolution mass spectra (HRMS) were recorded under ESI conditions on a BrukerMicroTOF (resolution = 5000 FWHM). The lock masses used for calibration were tetraoctylammonium bromide and sodium dodecyl sulfate in positive and negative ion mode respectively. Elemental analysis of solid compounds for C,H,N was obtained by the Mr Stephen Boyer, London Metropolitan University. Where elementals are not reported, these were metal compounds showing consistently low C and N values, indicative of the presence of H_2O. Single peak HPLC traces and high resolution mass spectrometry were obtained for these compounds.

Analytical HPLC spectra were recorded on a Gilson instrument with a C-18 column and UV/vis detection at 254 nm. Solvent conditions were as follows. Method I (M I) solvent A = H_2O + 0.1% TFA, solvent B = CH_3CN+ 0.1% TFA, flow rate = 1 ml min^{-1}, gradient (min,% of B): 0, 5; 15, 95; 20, 95; 25, 5; 30, 5. Method II (M II) solvent A = H_2O, solvent B = CH_3CN, flow rate = 1 ml min^{-1}, gradient (min,% of B): 0, 5; 15, 95; 20, 95; 25, 5; 30, 5. Analytical HPLC conditions for peptide conjugates are detailed below.

Synthesis of precursors

Proligands H_2ATSM/A and H_2ATSE/A (**1** and **2**) and the Zn complex **3** were synthesised according to previously reported procedures.[5]

Synthesis of linkers

General procedure A: Coupling reactions of H_2ATSE/A with carboxylic acid linkers. H_2ATSE/A was suspended in the minimum anount of DMF. The carboxylic acid (1.1 eq), diisopropylethylamine (1.1 eq) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (1.1 eq) were added and the mixture was stirred at room temperature for 4 h until a clear solution was formed. H_2O was then added until formation of a white precipitate. The suspension was sonicated, the precipitate collected by filtration and washed with copious amounts of H_2O, EtOH and, if solubility allowed, Et_2O.

General procedure B: Copper complexation. The proligand and $CuCl_2$ (1.2 eq) or $Cu(OAc)_2{\cdot}H_2O$ (1.2 eq) were stirred in a minimum amount of MeOH for 30 min at rt. The solvent was removed *in vacuo* and H_2O was added. The solid formed was collected by filtration and washed with H_2O and, where solubility allowed, with a few drops of ice-cold Et_2O before being dried *in vacuo*.

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N* (amino)-(4-carboxyphenylmethylidene)-3-thiosemicarbazone] (4a). **1** (500 mg, 1.82 mmol) was suspended in MeOH (20 ml). 4-formylbenzoic acid (354 mg, 3.33 mmol) was added and the suspension stirred overnight at rt. The residue was filtered and dissolved in DMF. MeOH was added to induce precipitation of **4a** (655 mg, 82%) as a pale yellow solid.

^{1}H NMR (300 MHz, DMSO-d_6): δ 13.02 (1H, s, COOH), 12.31 (1H, s, HC=NN*H*(C=S)NHN=), 10.74 (1H, s, HC=NNH(C=S)N*H*N=), 10.22 (1H, s, EtNH(C=S)N*H*N=), 8.48 (1H, t, J = 5.7 Hz, N*H*Et), 8.24 (1H, s, ArC*H*=N), 8.00 (2H, d, J = 7.9 Hz, ArC*H*), 7.86 (2H, d, J = 7.9 Hz, ArC*H*), 3.62 (2H, m, CH_2CH$_3$), 2.31 (3H, s, CH_3C=N), 2.25 (3H, s, CH_3C=N) 1.15 (3H, t, J = 7.3 Hz, CH$_2$CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.5 (EtNH*C*=S), 174.9 (HC=NNH(*C*=S), 166.8 (*C*O$_2$H), 153.4 (HC=NNH(C=S)NHN=*C*), 147.9 (EtNH(C=S)NHN=*C*), 142.1 (H*C*=NNH), 137.9 (Ar*C*), 131.7 (Ar*C*), 129.7 (Ar*C*H), 127.2 (Ar*C*H), 38.6 (*C*H$_2$CH$_3$), 14.3 (*C*H$_2$*C*H$_3$), 11.6 (*C*H$_3$C=NNH(C=S)NHEt), 11.5 (*C*H$_3$C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{20}N_7O_2S_2$ (M-H)$^-$ 406.1125; found 406.1137; HPLC (M I) R$_t$ 11.85 min; Elemental Analysis Found: C, 47.2; H, 5.1; N, 24.0. $C_{16}H_{21}N_7O_2S_2$ requires: C, 47.2; H, 5.2; N, 24.1%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-(2-carboxyphenylmethylidene)-3-thiosemicarbazone] (4b). was prepared according to the procedure described for **4a** using **1** (0.250 g, 0.91 mmol) and 2-formylbenzoic acid (177 mg, 1.18 mmol) in MeOH (20 ml) to afford **4b** (305 mg, 82%) as a pale yellow solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.85 (1H, s, COOH), 12.31 (1H, s, HC=NN*H*(C=S)NHN=), 10.66 (1H, s, HC=NNH(C=S)N*H*N=), 10.20 (1H, s, EtNH(C=S)N*H*N=), 8.88 (1H, s, ArC*H*=N), 8.46 (1H, t, J = 5.7 Hz, N*H*Et), 8.06 (1H, d, J = 7.62 Hz, ArCH-4 or ArCH-6), 7.86 (1H, d, J = 7.61 Hz, ArCH-3 or ArCH-6), 7.62 (1H, dt, J = 7.6 Hz, J = 8.5 Hz, ArCH-4 or ArCH-5), 7.52 (1H, dt, J = 7.6 Hz, J = 8.5 Hz, ArCH-4 or ArCH-5), 3.66-3.57 (2H, m, CH_2CH$_3$), 2.27 (3H, s, CH_3C=N), 2.23 (3H, s, CH_3C=N) and 1.13 (3H, t, J = 7.0 Hz, CH$_2$CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.8 (EtNH*C*=S), 177.9 (HC=NNH(*C*=S), 168.7 (*C*O$_2$H), 149.8 (HC=NNH(C=S)NHN=*C*), 147.9 (EtNH(C=S)NHN=*C*), 142.6 (H*C*=NNH), 136.7 (Ar*C*), 133.4 (Ar*C*), 129.4 (Ar*C*H), 129.3 (Ar*C*H), 127.8 (Ar*C*H), 124.4 (Ar*C*H) 38.6 (*C*H$_2$CH$_3$), 14.3 (CH$_2$*C*H$_3$), 11.9 (*C*H$_3$C=NNH(C=S)NHEt), 11.6 (*C*H$_3$C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{20}N_7O_2S_2$ (M-H)$^-$ 406.1125, found 406.1123; HPLC (M I) Rt 12.05 min, Elemental Analysis Found C, 47.1; H, 5.1; N, 24.0. $C_{16}H_{21}N_7O_2S_2$ requires C, 47.2; H, 5.2; N 24.1%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-(4-carboxyphenylmethylidene)-3-thiosemicarbazonato]zinc(II)) (5a). **4a** (70 mg, 0.172 mmol) was suspended in MeOH (5 ml) at rt. $ZnCl_2$ (28 mg, 0.206 mmol) was dissolved in the minimum amount of H_2O and added dropwise. The solution turned yellow immediately and was stirred for 3 h at rt. The solvent was then removed *in vacuo* and H_2O added. The resulting precipitate was filtered off, washed with cold Et_2O and dried *in vacuo* to afford the desired product (68 mg, 85%) as an orange solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.05 (1H, s, COOH), 8.49 (1H, t, J = 5.7 Hz, N*H*Et), 8.18-8.16 (1H, m, ArC*H*=N), 8.01-7.98 (2H, m, ArH), 7.87-7.76 (d, J = 8.2 Hz, ArH) 3.66-3.57 (2H, m, CH_2CH$_3$), 2.29 (3H, s, CH_3C=N), 2.23 (3H, s, CH_3C=N) and 1.15 (3H, t, J = 6.93 Hz, CH$_2$CH_3);^{13}C NMR (126 MHz, DMSO-d_6): δ 177.5 (*C*-S), *other* (*C*-S) *not observed*, 167.0 and 168.9 *two isomers* (*C*O$_2$H), (HC=NNH(C=S)NHN=*C*) *not observed*, 147.8 (EtNH(C=S)NHN=*C*), 142.2 (*H*C=NNH), 137.9 (Ar*C*) 131.8 (Ar*C*), 129.8 (Ar*C*H), 127.3 (Ar*C*H), 38.6 (*C*H$_2$CH$_3$), 14.4 (*C*H$_2$*C*H$_3$), 11.6 and 11.5 (2 × *C*H$_3$C=N); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{18}N_7O_2S_2Zn^-$ 468.0260; found 468.0267; HPLC (M I) Rt 11.75 min

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-(2-carboxyphenylmethylidene)-3-thiosemicarbazonato]zinc(II) (5b). **4b** (70 mg, 0.172 mmol) was suspended in MeOH (5 ml) at rt. $ZnCl_2$ (27.7 mg, 0.206 mmol) was dissolved in the minimum amount of H_2O and added dropwise. The solution turned yellow immediately and was stirred for 3 h at rt. The solvent was then removed *in vacuo* and H_2O added. The resulting precipitate was filtered off, washed with cold Et_2O and dried *in vacuo* to afford the desired product **8a** (73 mg, 91%) as an orange solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 13.12 (1H, s, COOH), 8.86 (1H, s, ArC*H*=N), 8.47 (1H, t, J = 5.7 Hz, N*H*Et), 8.07 (1H, d, J = 7.7 Hz, ArCH-3 or -6), 7.88 (1H, d, J = 7.7 Hz, 3-ArCH or 6-ArCH), 7.63 (1H, t, J = 7.3 Hz, ArCH-4 or H-5), 7.52 (1H, t, J = 7.3 Hz, ArCH-4 or H-5), 3.66-3.59 (2H, m, CH_2CH$_3$), 2.29 (3H, s, CH_3C=N), 2.23 (3H, s, CH_3C=N) and 1.15 (3H, t, J = 6.9 Hz, CH$_2$CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ (EtNH*C*=S) *not observed*, 177.4 (HC=NNH(*C*=S), 168.1 (*C*O$_2$H), (HC=NNH(C=S)NHN=*C*) *not observed*, 147.8 (EtNH(C=S)NHN=*C*), 142.5 (*H*C=NNH), 131.9 (Ar*C*), 131.7 (Ar*C*), 131.0 (Ar*C*H), 130.1 (Ar*C*H), 129.8 (Ar*C*H), 127.0 (Ar*C*H), 38.5 (*C*H$_2$CH$_3$), 14.3 (*C*H$_2$*C*H$_3$), 11.5 (*C*H$_3$C=N), 11.5 (*C*H$_3$C=N); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{18}N_7O_2S_2Zn^-$ 468.0260; found 468.0259; HPLC (M II) R$_t$ 11.75 min, Elemental Analysis Found C, 40.9; H, 4.1; N, 20.8. $C_{16}H_{19}N_7O_2S_2Zn$ requires C, 40.8; H, 4.1; N, 20.8%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-(4-carboxyphenylmethylidene)-3-thiosemicarbazonato]copper(II) (6a). **6a** was prepared following General Procedure B using **4a** (70 mg, 0.171 mmol) and $CuCl_2$ (28 mg, 0.177 mmol) in MeOH (5 ml) to yield the desired product as a brown solid (73 mg, 91%). HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{18}N_7O_2S_2Cu^-$ 467.0265; found 467.0253; HPLC (M I) Rt 12.25 min

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-(2-carboxyphenylmethylidene)-3-thiosemicarbazonato]copper(II) (6b). **6b** was prepared following General Procedure B using **4b** (60 mg, 0.147 mmol) and $CuCl_2$ (23.7 mg, 0.177 mmol) in MeOH (5 ml) to afford the desired product as a dark brown solid (66 mg, 96%).

HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{18}N_7O_2S_2Cu^-$ 467.0265; found 467.0255; HPLC (M I) R_t 12.05 min,; Elemental Analysis Found C, 40.9; H, 4.0; N, 20.9. $C_{16}H_{19}CuN_7O_2S_2$ requires C, 41.0; H, 4.1; N, 20.9%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-(4-tertbutoxycarboxybenzamide)-3-thiosemicarbazone] (7). **7** was synthesized according to General Procedure A, using H_2ATSE/A (300 mg, 1.1 mmol), 4-*tert*-butylbenzoic acid (266 mg, 1.2 mmol), BOP (530 mg, 1.2 mmol) and diisopropylethylamine (209 µl, 1.2 mmol). The product was isolated as an off-white solid (503 mg, 96%).

^{1}H NMR (500 MHz, DMSO-d_6): 10.78 (1H, s, N*H*(C=O)Ar), 10.71 (1H, s, NHNH(C=S)N*H*N=), 10.19 (1H, s, NHN*H*(C=S)NHN=), 10.17 (1H, s, EtNHC=SN*H*N=), 8.45 (1H, t, J = 5.8 Hz, N*H*CH_2CH_3), 8.02 (2H, d, J = 8.5 Hz, ArCH), 8.01 (2H, d, J = 8.5 Hz, ArCH), 3.66-3.59 (2H, m, J = 6.9 Hz, C$H_2$$CH_3$), 2.27 (3H, s, C$H_3$C=N), 2.25 (3H, s, C$H_3$C=N), 1.57 (9H, s, CO_2^{t}Bu), 1.15 (3H, t, J = 7.3 Hz, CH_2CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.8 (NHNH*C*=S), 177.4 (EtNH*C*=S), 166.7 (NHNH*C*=O), 164.9 (*C*O_2^{t}Bu), 149.8 (EtNH(C=S)NHN=*C*), 147.9 (NHNH(C=S)NHN=*C*), 136.7 (Ar*C*CO_2H), 133.9 (Ar*C*(C=O)NH), 129.3 (Ar*C*H), 127.8 (Ar*C*H), 81.3 (*C*$(CH_3)_3$), 38.6 (*C*$H_2$$CH_3$), 27.8 (C(*C*$H_3$)$_3$), 14.4 ($CH_2$*C*$H_3$), 11.9 (*C*$H_3$C=NNH(C=S)NHEt), 11.7 (*C*H_3C=NNH(C=S)NHNH); HRMS (ESI$^-$) (M+Na)$^+$ calcd for$^-$ $C_{20}H_{29}N_7NaO_3S_2$ 502.1666; found 502.1667; HPLC (MII) R_t 13.18 min Elemental Analysis Found C, 50.0; H, 6.0; N, 20.4. $C_{20}H_{29}N_7O_3S_2$ requires C, 50.1; H, 6.1; N 20.4%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-(4-carboxybenzamide)-3-thiosemicarbazone] (8). **7** (500 mg, 1.2 mmol) was suspended in TFA (2 ml) and stirred for 2.5 h at rt. TFA was then removed *in vacuo* and the resulting residue was sonicated in Et_2O, filtered and washed with more Et_2O. The resulting solid was suspended in warm EtOH and sonicated, filtered and dried to afford **8** (300 mg, 65%) as an off-white solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 13.18 (1H, br s, COOH), 10.78 (1H, br s, N*H*(C=O)Ar), 10.70 (1H, s, NHNH(C=S)N*H*N=), 10.20 (1H, s, NHN*H*(C=S)NHN=), 10.16 (1H, s, EtNHC=SN*H*N=), 8.45 (1H, t, J = 5.8 Hz, N*H*Et), 8.07 (2H, d, J = 8.5 Hz, ArC*H*), 8.01 (2H, d, J = 8.5 Hz, ArC*H*), 3.61 (2H, m, C$H_2$$CH_3$), 2.27 (3H, s, C$H_3$C=N), 2.25 (3H, s, C$H_3$C=N), 1.15 (3H, t, J = 7.3 Hz, CH_2CH_3); ^{13}C NMR (126 MHz, DMSO-d_6): δ 179.8 (NHNH*C*=S), 177.4 (EtNH*C*=S), 166.7 (NHNH*C*=O), 164.9 (*C*O_2H), 149.8 (EtNH(C=S)NHN=*C*), 147.9 (NHNH(C=S)NHN=*C*), 136.7 (Ar*C*CO_2H), 133.4 (Ar*C*(C=O)NH), 129.3 (Ar*C*H), 127.8 (Ar*C*H), 38.6 (*C*$H_2$$CH_3$), 14.3 ($CH_2$*C*$H_3$), 11.9 (*C*$H_3$C=NNH(C=S)NHEt), 11.6 (*C*H_3C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{16}H_{20}N_7O_3S_2^-$ 422.1075; found 422.1076; HPLC (M I) R_t 10.35 min; Elemental Analysis Found C, 45.3; H, 5.0; N, 23.2. $C_{16}H_{21}N_7O_3S_2$ requires C, 45.4; H 5.0; N, 23.2%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-(4-carboxybenzamide)-3 thiosemicarbazonato]copper(II) (9a). **9a** was prepared following General Procedure B, using **8** (38 mg, 0.09 mmol) and $CuCl_2$·H_2O (0.1 mmol, 1.1 eq) to afford the desired product as a red-brown solid (37 mg, 85%). HRMS (ESI$^+$): (M-H)$^-$ calcd for $C_{16}H_{18}CuN_7O_3S_2^-$ 483.0214; found 483.0213; HPLC (M I) R_t 10.30 min, Elemental Analysis Found C, 39.6; H, 4.0; N, 20.1. $C_{16}H_{19}CuN_7O_3S_2$ requires C, 39.6; H, 4.0; N, 20.2

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-methyl 2-(4-formylbenzamido)acetate)-3 thiosemicarbazone] (11a). **1b** (230 mg, 0.62 mmol) and methyl 2-(4-formylbenzamido) acetate (230 mg, 1.11 mmol) were stirred in MeOH (15 ml) overnight. The suspension was then heated at 50 °C for 2 h. The solvent was removed *in vacuo* and the solid was washed with cold MeOH (10 ml) and H_2O (10 ml) and dried to afford **3c** (195 mg, 68%). Methyl 2-(4-formylbenzamido) acetate was synthesised following a previously reported procedure.[14]

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.64 (brs, COOH), 12.3 (N*H*N=CH), 10.75 and 10.23 (2 × 1H, s, C=SN*H*-N=), 8.94 (1H, t, J = 5.7 Hz, N*H*CH_2CO_2H), 8.49 (1H, t, J = 5.8 Hz, N*H*Et), 8.26 (1H, brs, NHN=C*H*), 7.95 (2H, d, J = 8.2 Hz, ArC*H*), 7.85 (2H, d, J = 7.9 Hz, ArC*H*), 3.96 (2H, d, J = 5.7 Hz, C$H_2$$CO_2H$), 3.66-3.57 (2H, m, C$H_2$$CH_3$), 2.31 (3H, s, C$H_3$C=N), 2.25 (3H, s, C$H_3$C=N), 1.15 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (126 MHz, DMSO-d_6): δ 177.5 (EtNH*C*=S), 175.1 (*C*=S), 171.3 (*C*O_2H), 165.9 (*C*ONHCH$_2$), 153.3 (NHNH(C=S)NHN=*C*), 147.8 (EtNH(C=S)NHN=*C*), 142.1 (H*C*=NNH), 136.8 (Ar*C*), 134.8 (Ar*C*), 127.8 (Ar*C*H), 127.1 (Ar*C*H), 41.3 (*C*$H_2$$CO_2H$), 38.6 (*C*$H_2$$CH_3$), 14.4 (*C*$H_2$$CH_3$), 11.6, 11.1 (*C*$H_3$C=NNH(C=S)NHEt), (*C*$H_3$C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M+Na)$^+$ calcd for $C_{18}H_{24}NaN_8O_3S_2^+$ 487.1305; found 487.1306; Elemental Analysis Found C, 46.6; H, 5.2; N, 24.1. $C_{18}H_{24}N_8O_3S_2$ requires C, 46.5; H, 5.2; N 24.1%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-4(*N*-(2-*tert*butylacetate)benzamide))-3-thiosemicarbazone (11b). **11b** was synthesised according to General Procedure A using **8** (250 mg, 0.59 mmol), Glycine *tert*-butyl ester hydrochloride (110 mg, 0.65 mmol), diisopropylethylamine (168 mg, 1.30 mmol) and BOP (288 mg, 0.65 mmol) in DMF (5 ml). The product was isolated as an off-white solid (283 mg, 98%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 10.78 (2H, br s, (C=S)NHN*H*-C=O) and (C=S)N*H*NH–C=O), 10.17 (2H, brs s, 2 × (C=S)-NH–N), 9.00 (1H, t, J = 5.8 Hz, N*H*CH_2CO_2^{t}Bu), 8.45 (1H, t, J = 5.8 Hz, N*H*Et), 8.01 (2H, d, J = 8.2 Hz, ArC*H*), 7.98 (2H, d, J = 8.5 Hz, ArC*H*), 3.92 (2H, d, J = 5.8 Hz, C$H_2$$CO_2$^{t}Bu), 3.66-3.57 (2H, m, C$H_2$$CH_3$), 2.27 (3H, s, C$H_3$C=N), 2.25 (3H, s, C$H_3$C=N), 1.43 (9H, s, CO_2^{t}Bu), 1.15 (3H, t, J = 6.6 Hz, CH_2CH_3); ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 179.7 (NHNH*C*=S), 177.4 (EtNH*C*=S), 168.7 (*C*O_2^{t}Bu), 165.8 (*C*ONHCH$_2$), 164.9 (*C*ONHNH), 149.7 (EtNH(C=S)NHN=*C*), 147.9 (NHNH(C=S)NHN=*C*), 136.5 (Ar*C*), 133.4 (Ar*C*), 127.6 (Ar*C*H), 127.3 (Ar*C*H), 80.72 (*C*(CH_3), 41.9 (*C*$H_2$$CO_2$^{t}Bu), 38.6 (*C*$H_2$$CH_3$), 27.7 (C(*C*$H_3$), 14.3 ($CH_2$*C*$H_3$), 11.9 (*C*$H_3$C=NNH(C=S)NHEt), 11.6 (*C*H_3C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{22}H_{31}N_8O_4S_2^-$ 535.1915; found 535.1921; HPLC (M I) Rt 11.8 min Elemental Analysis Found C, 49.4; H, 6.1; N, 20.9. $C_{22}H_{32}N_8O_4S_2$ requires C, 49.2; H, 6.0; N 20.9%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazone)-3-[4-*N*-(amino)-4(*N*-(2-acetic acid)benzamide)-3-thiosemicarbazone (11c). **11b** (280 mg, 0.052 mmol) was suspended in TFA (3 ml) and stirred at room temperature for 2.25 h. TFA was then removed *in vacuo* and

the resulting residue was sonicated in Et_2O, filtered and washed with more Et_2O and dried to afford the desired product (178 mg, 71%) as a pale yellow solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.59 (brs, COOH), 10.74-10.70 (2H, br s, (C=S)NHN*H*-C=O) and (C=S)N*H*NH–C=O), 10.17 (2H, brs s, 2 × (C=S)-NH–N), 8.99 (1H, t, J = 5.8 Hz, N*H*CH_2CO_2H), 8.45 (1H, t, J = 5.8 Hz, N*H*Et), 8.01 (2H, d, J = 8.2 Hz, ArC*H*), 7.98 (2H, d, J = 8.5 Hz, ArC*H*), 3.96 (2H, d, J = 5.8 Hz, CH_2CO_2H), 3.65-3.57 (2H, m, CH_2CH_3), 2.27 (3H, s, CH_3C=N), 2.25 (3H, s, CH_3C=N), 1.15 (3H, t, J = 6.6 Hz, CH_2CH_3); ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 179.8 (NHNH*C*=S), 177.4 (EtNH*C*=S), 171.17 (*C*O_2H), 165.8 (*C*ONHCH$_2$), 164.9 (*C*ONHNH), 149.7 (EtNH(C=S)NHN=*C*), 147.9 (NHNH(C=S)NHN=*C*), 136.5 (Ar*C*), 135.4 (Ar*C*), 127.6 (Ar*C*H), 127.3 (Ar*C*H), 41.2 (NH*C*H_2CO_2H) 38.6 (*C*H_2CH_3), 14.4 (*C*H_2*C*H_3), 11.9 (*C*H_3C=NNH(C=S)NHEt), 11.6 (*C*H_3C=NNH(C=S)NHNH); HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{18}H_{23}N_8O_4S_2^-$ 479.1289; found 479.1278; HPLC (M I) Rt 9.48 min Elemental Analysis Found C, 44.9; H, 5.1; N, 23.4. $C_{18}H_{24}N_8O_4S_2$ requires C, 50.0; H, 5.0; N, 23.3%

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-Methyl 2-(4-formylbenzamido)acetate)-3 thiosemicarbazonato]-copper(II) (12a). **12a** was prepared following General Procedure B, using **11a** (30 mg, 0.06 mmol) and $CuCl_2$ (12 mg, 0.07 mmol) to afford the desired product as a black solid (25 mg, 76%). HRMS (ESI$^+$): (M-H)$^-$ calcd for $C_{18}H_{19}CuN_7O_3S_2^-$ 522.0323; found 522.0317; HPLC (M I) R_t 10.30 min

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-[4-*N*-(amino)-4(*N*-(2-acetic acid)benzamide)-3-thiosemicarbazonato]copper(II) (12c). **12c** was prepared following General Procedure B, using **11c** (60 mg, 0.125 mmol) and $Cu(OAc)_2{\cdot}H_2O$ (33 mg, 1.3 eq) to afford the desired product as a black solid (65 mg, 96%). HRMS (ESI$^-$): (M-H)$^-$ calcd for $C_{18}H_{21}CuN_8O_4S_2^-$ 540.0418; found 540.0429; HPLC (M I) Rt 9.07 min

H_2ATSM/A-α-Fmoc-L-Glu(O^{t}Bu) (14). **14** was prepared according to General Procedure A using **2** (543 mg, 2.08 mmol), Fmoc*L*-GluO^{t}Bu (885 mg, 2.08 mmol), diisopropylethylamine (362 µl, 2.08 mmol) and BOP (920 mg, 2.08 mmol) in DMF (7 ml). The precipitate was then stirred in hot EtOH (5-10 ml) before being filtered off and washed with cold Et_2O (10 ml) to afford 975 mg (70%) of **14** as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.60 (1H, s, NHNH(C=S)N*H*N=), 10.25 (1H, s, MeNH(C=S)N*H*N=), 10.20 (1H, s, N*H*NH(C=S)NHN=), 10.02 (1H, s, NHN*H*(C=S)NHN=), 8.40 (d, 1H, J = 4.5 Hz, CH_3N*H*), 7.90 (2H, d, J = 7.5 Hz, H-4, H-5), 7.75 (2H, m, H-1, H-8), 7.62 (1H, d, J = 8.88 Hz, N*H*Fmoc), 7.42 (2H, t, J = 7.5 Hz, H-3, H-6), 7.33 (2H, dt, J = 7.5 Hz, J = 1.4 Hz, H-2, H-7), 4.27-4.17 (4H, m, CH_α, CH_2Fmoc, H-9), 3.02 (3H, d, J = 4.5 Hz, CH_3NH), 2.37 (2H, t, J = 6.6 Hz, CH_γ), 2.23 (3H, s, CH_3C=N), 2.21 (3H, s, CH_3C=N), 2.08 (1H, m, CH_β), 1.85 (1H, m, CH_β), 1.14 (9H, s, C(CH_3)); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.2 (NHNH*C*=S), 178.4 (EtNH*C*=S), 171.7 (*C*O_2^{t}Bu), 170.1 (NHNH*C*O), 155.8 ((*C*=O)Fmoc), 149.7 (NHNH(C=S)NH(*C*=N)), 147.8 MeNH(C=S)NH(*C*=N), 143.8 (Ar*C*), 143.7 (Ar*C*), 140.6 (Ar*C*), 127.6 (C^{-3}, C-6), 127.0 (C^{-2}, C-7), 125.3 (C^{-1}, C-8), 120.1 (C^{-4}, C-5), 79.5 (*C*CH_3), 65.7 (*C*H_2Fmoc), 52.4 (C_α), 46.6 (C-9), 31.4 (C_γ), 31.2 (NH*C*H_3), 27.7 (C(*C*H_3)), 27.5 (C_β), 11.8 (*C*H_3(C=N)NH(C=S)NHNH), 11.6 (*C*H_3(C=N)NH(C=S)NHMe); HRMS (ESI$^+$): (M+Na)$^+$ calcd for $C_{31}H_{40}N_8NaO_5S_2^+$ 691.2455; found 691.2437; HPLC (MII) R_t 14.55 min; Elemental Analysis Found C, 55.6; H, 5.9; N, 16.6. $C_{31}H_{40}N_8O_5S_2$ requires C, 55.7; H, 5.9; N, 16.6%

H_2ATSM/A-α-NH$_2$-L-Glu(O^{t}Bu) (15). **14** (100 mg, 0.150 mmol) was stirred in DMF (600 µl) and piperidine (120 µl) for 45 min. The solution was concentrated and H_2O was added until the formation of a white precipitate. The white precipitate was filtered off, washed with a few more drops of H_2O. The aqueous phase was concentrated and Et_2O was added until formation of a precipitate. Filtration and drying under vacuum afforded **15** as an off-white solid (40 mg, 60%).

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.20 (4H, superimposed broad singlets, MeNH(C=S)N*H*N=, NHNH(C=S)N*H*N=, N*H*NH(C=S)NHN=, NHN*H*(C=S)NHN=), 8.40 (d, 1H, J = 4.7 Hz, CH_3N*H*), 3.33 (*obscured by* H_2O, CH_α) 3.02 (3H, d, J = 4.7 Hz, CH_3NH), 2.37 (2H, t, J = 6.6 Hz, CH_γ), 2.23 (3H, s, CH_3C=N), 2.21 (3H, s, CH_3C=N), 1.94-1.87 (1H, m, CH_β), 1.75-1.69 (1H, m, CH_β), 1.14 (9H, s, C(CH_3)); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.2 (NHNH*C*=S), 178.4 (EtNH*C*=S), 171.7 (*C*O_2tBu), 170.1 (NHNH*C*O), 149.5 (NHNH(C=S)NH(*C*=N)), 147.8 MeNH(C=S)NH(*C*=N), 79.5 (*C*CH_3), 52.5 (C_α), 31.4 (C_γ), 31.2 (NH*C*H_3), 27.7 (C(*C*H_3)), 27.5 (C_β), 11.8 (*C*H_3(C=N)NH(C=S)NHNH), 11.6 (*C*H_3(C=N)NH(C=S)NHMe); HRMS (ESI$^+$): (M+H)$^+$ calcd for $C_{16}H_{31}N_8O_3S_2^+$ 447.1955; found 447.1964; HPLC (M II) R_t 12.40 min Elemental Analysis Found C, 42.9; H, 6.8; N, 25.0. $C_{16}H_{30}N_8O_3S_2$ requires C, 43.0; H, 6.8; N, 25.1%

H_2ATSM/A-α-NH-Biotin-L-Glu(O^{t}Bu) (16). **16** was synthesised according to General Procedure A using **15** (40 mg, 0.089 mmol), biotin (23 mg, 0.09 mmol), diisopropylethylamine (16 µl, 0.09 mmol) and BOP (42 mg, 0.09 mmol) in DMF (5 ml). The product was isolated as a beige solid (38.2 mg, 63%).

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.58 (1H, s, NHNH(C=S)N*H*N=), 10.23 (1H, s, MeNH(C=S)N*H*N=), 10.17 (1H, s, N*H*NH(C=S)NHN=), 10.00 (1H, s, NHN*H*(C=S)NHN=), 8.41 (d, 1H, J = 4.5 Hz, CH_3N*H*), 7.89 (2H, d, J = 7.5 Hz, H-4, H-5), 7.75 (2H, m, H-1, H-8), 7.99 (1H, d, J = 8.5 Hz, N*H*biotin), 6.41 and 6.35 (2 × 1H, s, C*H*NHC=O), 4.45 (1H, dd, J = 13.8 Hz, J = 8.5 Hz, NHNH(C=O)C*H*), 4.30-4.13 (2H, m, 2×CHN*H*(C=O), 3.09 (1H, m, CH_2CHSCH_2), 3.02 (3H, d, J = 4.6 Hz, CH_3NH), 2.81 (1H, dd, J = 12.6 Hz, J = 5.2 Hz, 1×SCH_2CH), 2.54 (1H, m, obscured by DMSO, 1×SCH_2CH),2.36-2.31 (2H, $CH_2CH_2CO_2$^{t}Bu), 2.23 (3H, s, CH_3C=N), 2.21 (3H, s, CH_3C=N), 2.15-2.13 (2H, m, (C=O)CH_2(CH$_2$)$_3$), 2.04-1.24 (8H,m, (C=O)CH$_2$(CH_2)$_3$ and CH_2CH$_2$CO$_2$^{t}Bu), 1.40 (9H,s, CO_2*tBu*); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.21 (NHNH*C*=S), 178.47 (EtNH*C*=S), 172.03 (CO), 171.7 (*C*O_2tBu), 170.11 (NHNH*C*O), 162.66 (NH(C=O)NH), 149.78 (NHNH(C=S)NH(*C*=N)), 147.92 MeNH(C=S)NH(*C*=N), 79.57 ($CO_2C(CH_3)_3$), 61.01 (CH*C*HNH(C=O)), 59.18 (SCH$_2$*C*HNH(C=O)), 55.41 (*C*HS), 50.02 (*C*HCH$_2$CH$_2$CO$_2$^{t}Bu), 34.8 (NH(C=O)*C*H$_2$(CH$_2$)$_3$), 28.14 (CH_2), 28.01 (CH_2), 27.78 ($CO_2C(CH_3)_3$, 25.24 (CH_2), 11.84 (*C*H$_3$(C=N)NH(C=S)NHNH), 11.65 (*C*H$_3$(C=N)NH(C=S)NHMe); HRMS (ESI$^+$): (M+Na)$^+$

calcd for $C_{26}H_{44}N_{10}NaO_5S_3^+$ 695.2550; found 695.2554; HPLC R_t (M II) 11.26 min, Elemental Analysis Found C, 46.4; H, 6.5; N, 20.9. $C_{26}H_{44}N_{10}O_5S_3$ requires C, 46.4; H, 6.6; N, 20.8%

H_2ATSM/A-α-Fmoc-L-Glu(OH) (17). 14 (400 mg, 0.60 mmol) was stirred in TFA (3 ml) at room temperature for 3 h. The solvent was then evaporated *in vacuo*. Et_2O was added and the suspension sonicated before evaporating to dryness. The residue was stirred in warm EtOH (5 mL) and left to cool before being filtered, washed with Et_2O and dried *in vacuo* to afford **17** (216 mg, 59%) as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.61 (1H, s, NHNH(C=S)N*H*N=), 10.25 (1H, s, MeNH(C=S)N*H*N=), 10.20 (1H, s, N*H*NH(C=S)NHN=), 10.03 (1H, s, NHN*H*(C=S)NHN=), 8.41 (d, 1H, J = 4.6 Hz, CH_3N*H*), 7.89 (2H, d, J = 7.5 Hz, H-4, H-5), 7.75 (2H, m, H-1, H-8), 7.65 (1H, d, J = 8.2 Hz, N*H*Fmoc), 7.42 (2H, t, J = 7.5 Hz, H-3, H-6), 7.33 (2H, t, J = 7.0 Hz, H-2, H-7), 4.27-4.17 (4H, m, CH_α, C*H*$_2$Fmoc, H-9), 3.03 (3H, d, J = 4.6 Hz, C*H*$_3$NH), 2.42 (2H, t, J = 6.6 Hz, CH_γ), 2.23 (3H, s, C*H*$_3$C=N), 2.21 (3H, s, C*H*$_3$C=N), 2.10 (1H, m, C*H*$_\beta$), 1.89 (1H, m, C*H*$_\beta$); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.27 (NHNH*C*=S), 178.50 (EtNH*C*=S), 174.06 (*C*O$_2$H), 170.36 (NHNH*C*O), 155.90 ((*C*=O)Fmoc), 149.78 (NHNH(C=S)NH(*C*=N)), 147.92 MeNH(C=S)NH(*C*=N), 143.92 (Ar*C*), 143.79 (Ar*C*), 140.73 (Ar*C*), 127.70 (C^{-3}, C-6), 127.12 (C^{-2}, C-7), 125.41 (C^{-1}, C-8), 120.15 (C^{-4}, C-5), 65.81 (*C*H$_2$Fmoc), 52.57 (C_α), 46.67 (C-9), 31.28 (C_γ), 30.39 (NH*C*H$_3$), 27.54 (C_β), 11.83 (*C*H$_3$(C=N)NH(C=S)NHNH), 11.64 (*C*H$_3$(C=N)NH(C=S)NHMe); HRMS (ESI$^+$): (M-H)$^-$ calcd for $C_{27}H_{33}N_8O_5S_2^+$ 613.2010; found 613.1989; HPLC (M I) R_t 12.45 min, Elemental Analysis Found C, 52.9; H, 5.2; N, 18.2 $C_{27}H_{32}N_8O_5S_2$ requires C, 52.9; H, 5.3; N, 18.3%

CuATSM/A-α-*L*-FmocGlu(OH) (18). $Cu(OAc)_2{\cdot}H_2O$ (12 mg, 0.059 mmol) was dissolved in H_2O (1 ml) and added dropwise to a stirring suspension of **15** (30 mg, 0.048 mmol) in MeOH (5 ml). The suspension turned brown immediately and was left to stir for 1 h. The solvent was then removed *in vacuo* and H_2O was added, the residue was filtered, washed with cold Et_2O and dried *in vacuo* to afford **18** (25 mg, 77%) as a dark brown solid. HPLC (M I) R_t 12.40 min, HRMS (ESI$^+$): (M-H)$^-$ calcd for $C_{27}H_{29}CuN_8O_5S_2^-$ 672.1004; found 672.0983

CuATSM/A-α-*N*-Biotin-L-Glu(O^{t}Bu) (19). 19 was prepared following General Procedure B, using **16** (38 mg, 0.056 mmol) and $Cu(OAc)_2{\cdot}H_2O$ (14 mg, 1.2 eq) to afford the desired product as a black solid (40 mg, 96%). HRMS (ESI$^+$): (M+Na)$^+$ calcd for $C_{26}H_{42}CuN_{10}NaO_5S_3^+$ 756.1690; found 756.1691; HPLC (M II) R_t 11.84 min

Synthesis of the BBS-conjugates

The synthesis of the Bombesin analogues was performed manually as previously described[24,25] by solid-phase peptide synthesis on a Fmoc Rink Amide resin (Novabiochem). The synthesis was carried out in a 2.5 mL plastic syringe fitted with a PE frit (MultiSynTech GmbH, Germany) with a maximum of 50 mg Fmoc-Rink Amide resin per syringe. The Fmoc group was removed by treatment with 25% 4-methylpiperidine in DMF (2 × 10 min), the resin was washed with 3 × DMF, 3 × ^{i}PrOH and 3 × DMF. Unless mentioned otherwise, all couplings were carried out in duplicate (2 × 90 min) with 4 eq Fmoc-amino acid, 4 eq DIC and 4 eq HOBt in DMF. A stock solution of HOBt in DMF was used to dissolve the amino acid ([AA] = 0.5 mM). After each coupling the resin was washed with 3 × DMF, 3 × ^{i}PrOH and 3 × DMF. The completion of each coupling was checked with the NF31 color test. Chelators **8** (2 eq) and **11c** (2 eq) were coupled in a single coupling to β^{3}hAsp using 2 eq of BOP, 2 eq diisopropylethylamine (8 h) or 2 eq DIC and 2 eq HOBt (3 h) respectively. At the end of the synthesis the resin was washed several times with CH_2Cl_2 and Et_2O and dried *in vacuo* before cleavage.

For cleavage from the resin, a mixture of thioanisole (TA)/ethanedithiol (EDT) 7 : 3 was added as scavenger to the TFA. The ligand- peptide-resin (0.2 mmol) was shaken in 900 µl TFA and 100 µl TA/EDT in a syringe fitted with a glass frit for 3.5 h. The filtrate was added dropwise to ice-cooled Et_2O (10 ml) and the mixture was left in an ice-bath for 15 min before centrifugation for 5 min. The Et_2O was decanted and the solid resuspended in Et_2O, left to settle in the ice-bath and centrifuged again. This operation was repeated twice more to eliminate all remaining TFA and scavengers. The resulting precipitate was dried, dissolved in CH_3CN–H_2O with 5% DMSO and lyophilized.

Analytical HPLC for BBS-conjugates was performed on a Waters Breeze™ HPLC system with UV detection (215 nm) using an RP-column (Supelco Discovery Bio Wide Pore, C 18, 5 µm, 250 mm × 4.6 mm). Gradient 1: 3% B to 100%B in 30 min, flow rate 1 ml min^{-1}, the gradient was finished by washing with 100% B. (solvent B = 0.1% TFA in CH_3CN).

Analysis was performed by coupling the mass spectrometer to a Waters system equipped with a UV detector (Waters, 215 nm), a manual injector and Waters 600E pump. The runs were performed on an RP-column (Supelco Discovery Bio Wide Pore, C 18, 5 µm, 250 mm × 4.6 mm) at a flow rate of 1 ml min^{-1}. The outlet of the HPLC column passed through a splitter with a split ratio of 1/10.

Preparative HPLC was carried out on a Gilson System fitted with a reverse phase C18 Column, Supelco Discovery Bio Wide Pore, 10 m, 250 mm × 21.2 mm, flow rate 20 ml min^{-1}.

Electron paramagnetic resonance

CW EPR experiments were performed on an X-band Bruker BioSpin GmbH EMX spectrometer equipped with a high sensitivity Bruker probehead and a low-temperature Oxford Instruments CF935 helium-flow cryostat. The magnetic field was calibrated at room temperature with an external 2,2-diphenyl-1-picrylhydrazyl standard ($/g/$ = 2.0036). X-band CW experiments were conducted at 60 K, with 0.20 mW microwave power, and 2.0 G modulation amplitude at 100 kHz.

Radiolabeling and octanol/water partition coefficients of ATSM-BBS conjugates

20 µl of 1 mM stock solution ATSM-BBS-1 or 2 in DMSO were transferred into 280 µl of 0.25 M ammonium acetate at pH 5.5. 2.5 µl $^{64/67}$Cu (equivalent to 0.045µg Cu) was added and the solution was shaken for 30 min at room temperature. The labeling was purified on a Sep-Pak™ C_{18} or Chromafix® C_{18}ec. by washing with 2 ml H_2O and eluting with 2 × 1 ml EtOH–H_2O 9 : 1.

The octanol/water partition coefficients were determined at pH 7.4 by adding 5 µl of radiolabeled **20** and **21** in PBS to a vial

containing 1.2 mL of 1 : 1 octanol:PBS. Vortex mixing for 1 min was followed by centrifugation at 10 000 rpm for 5 min. 40 µl from each separated layer were sampled into a pre-weighed vial and measured in a γ-counter. Counts per unit weight of sample were calculated and log D values were obtained using the formula log $D = \log$ (counts in 1 g of octanol/counts in 1 g of water).

Cell culture

The human prostate adenocarcinoma cell line PC-3 was purchased from the European Collection of Cell Culture (ECACC, Salisbury, UK). Cells were maintained in DMEM GLUTAMAX-I (Invitrogen) supplemented with 1-10% FCS, 100 IU/ml penicillin G sodium, 100 µg ml^{-1} streptomycin sulfate and 0.25µg ml^{-1} amphotericin B (Bioconcept). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. The cells were subcultured weekly after detaching them with trypsin/EDTA 0.25% (Invitrogen).

Binding assays

The binding assays were carried out as previously reported.[36] All experiments were carried out twice in triplicate. PC-3 cells were placed in 48-well plates (250,000 cells/well). A protease inhibitor containing binding buffer was used (50 mM HEPES, 125 mM NaCl, 7.5 mM KCl, 5.5 mM $MgCl_2$, 1 mM EGTA, 5 g l^{-1} BSA, 2 mg l^{-1} chymostatin, 100 mg l^{-1} soybean trypsin inhibitor and 50 mg l^{-1} bacitracin). Cells were incubated at 37 °C with 15,000-25,000 cpm of [^{125}I-Tyr^4]BBS per well and increasing concentrations (0-3000nM) of the non-labeled ATSM-BBS analogues. After 1 h incubation the cells were rinsed twice with cold PBS. Cells with bound activity were solubilised by adding 2 × 400 µl of 1 M NaOH at 37 °C. The final suspension was measured in a γ-counter. IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism.

Internalization assays

The internalisation assays were carried out as previously reported.[36] PC-3 cells at confluence were placed in 6-well plates at ~10^6 cells/well. Cells were incubated with ~1 kBq of the $^{64/67}$Cu-labeled analogues in culture medium at a total volume of 1 ml/well for 5, 15, 30, 60 and 120 min at 37 °C to allow binding and internalisation. Non-specific internalisation was determined in the presence of 1 µM non-labeled BBS(7–14). After each incubation time, cells were washed three times with PBS to remove any unbound peptide. Surface-bound activity was removed by two steps of 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH2.8, 600 µl per well) at room temperature. Cells containing internalised activity were then solubilised by adding 2 × 600 µl 1 M NaOH/well. Surface bound and internalised activity were measured in a γ-counter. The data reported refers to the specific internalisation after subtracting the non-specific binding and are given as a percentage of the total activity added per milligram of protein.

Biodistribution studies

All animal experiments were conducted in compliance with the Swiss animal protection laws and guidelines for scientific animal trials established by the Swiss Academy of Medical Sciences and the Swiss Academy of Natural Sciences. Female CD-1 nu/nu mice 6-8 week-old (Charles River Laboratories, Sulzfeld, Germany) were subcutaneously injected with 8×10^6 PC-3 cells. Three weeks after tumour implantation, the mice received i.v. 100 kBq $^{64/67}$Cu-ATSM-BBS-2. At 1 and 24 h postinjection (p.i.), the animals were killed by cervical dislocation. Blood and different organs were collected, weighed, and the radioactivity measured in a γ-counter. Results are presented as percentage of injected dose per gram of tissue (%ID/g). To determine the specificity of the *in vivo* uptake, one group of mice received a co-injection of 100 µg of unlabeled BBS(1–14) and the radiolabeled analogue and were sacrificed at 1 h p.i.

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