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Synthesis and activity of a folate peptide camptothecin prodrug

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Abstract—A folate receptor targeted camptothecin prodrug was synthesized using a hydrophilic peptide spacer linked to folate via a releasable disulfide carbonate linker. The conjugate was found to possess high affinity for folate receptor-expressing cells and inhibited cell proliferation in human KB cells with an IC_{50} of 10 nM. Activity of the prodrug was completely blocked by excess folic acid, demonstrating receptor-mediated uptake.

Tumor-targeted chemotherapeutic agents are attracting increased attention because of their ability to decrease toxicity to nonmalignant cells.[1–5] One of the more attractive molecular targets to emerge in this area is the folate receptor (FR), because (i) it is over-expressed on many tumors, including cancers of the breast, lung, kidney, ovary, brain, and myelogenous cells, and (ii) it is present in low or non-detectable quantities in most normal tissues.[6–12] Moreover, the vitamin folic acid and its drug conjugates bind FR with nanomolar affinity and enter cancer cells by receptor-mediated endocytosis.[13,14] Thus, the development of folate-tethered gene therapy vectors,[15] immunogenic haptens,[16] protein toxins,[17,18] liposomes,[19] imaging agents,[20–22] and low molecular weight drugs[5,23,24] has proceeded rapidly.

Camptothecin (CPT), originally isolated from the Chinese tree *Camptotheca acuminate*, possesses potent antitumor properties that derive from its inhibition of topoisomerase I.[25–27] However, clinical use of CPT (**1**) has been severely hindered by toxicity stemming in part from the instability of its *E*-lactone ring (Fig. 1), resulting in formation of the inactive but toxic carboxylate species (**2**).[28,29] In addition, problems with delivery due to poor water solubility have plagued development of CPT as a therapeutic agent.[26,28,30]

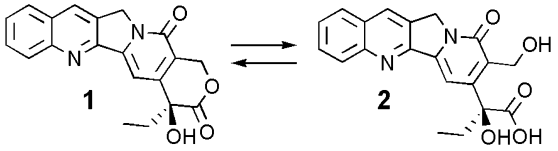

Figure 1. Camptothecin in its closed (1) and lactone ring opened form (2).

Ever since conjugation to the 20-OH of camptothecin was demonstrated to stabilize the lactone ring, prodrugs derivatized at this site have been vigorously pursued.[29,31,32] Simultaneous efforts to improve water solubility have led researchers to attach large PEG (polyethylene glycol) adducts that can be readily released by ester hydrolysis.[33] Our approach to the above chemical stability/water solubility problems has been to construct a folate conjugate of CPT linked to the drug at its 20-OH via a hydrophilic peptide spacer containing a disulfide releasable carbonate linker that together provide: (i) enhanced water solubility, (ii) minimal interference with folate-receptor binding, and (iii) efficient release of unmodified camptothecin via endosomal disulfide reduction.

Synthesis of the disulfide cleavable CPT prodrug was initiated (Scheme 1) by construction of the 2-(2-pyridinyldithio)-ethanol arm (**5**). 2-Mercaptoethanol (**3**) was reacted with 2,2′-dipyridyl disulfide (**4**) to generate **5**.[34] The mixed disulfide afforded reactive groups for subsequent coupling to both a folate-peptide thiol construct and for forming a carbonate bridge that connects to the 20-OH of CPT. Based on published data,[28,29,32]

Keywords: Folate; Folic acid; Camptothecin; Targeted drug delivery; Endocytosis; Releasable linker; Peptide; Prodrug; Carbonate; Disulfide.
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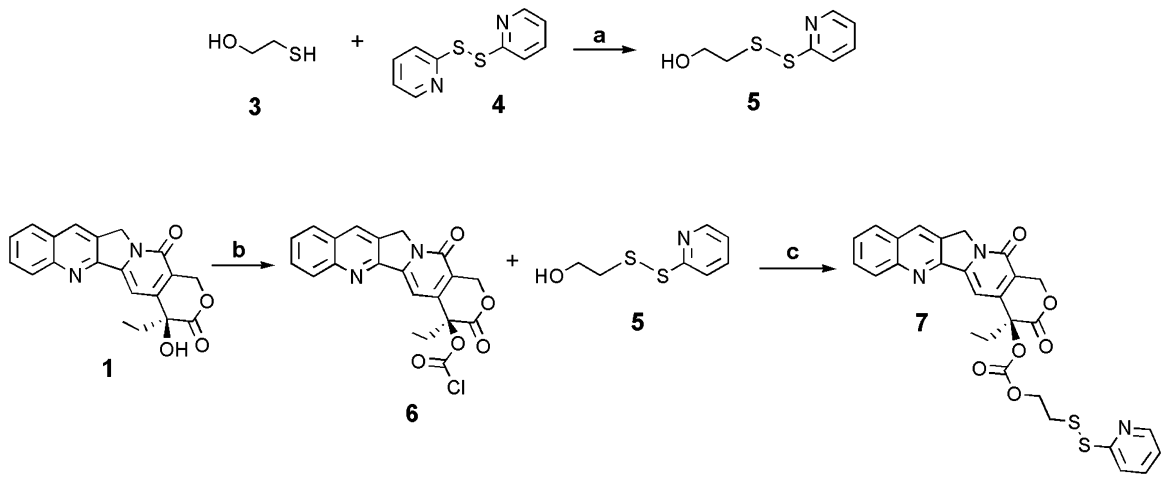


Scheme 1. Synthesis of pyridyldithioethyl carbonate camptothecin. Reagents and conditions: (a) CH_2Cl_2 rt, 30 min; (b) 0.35 equiv triphosgene, 6 equiv DMAP, CH_2Cl_2 rt, 15 min; (c) CH_2Cl_2 rt, 6 h.

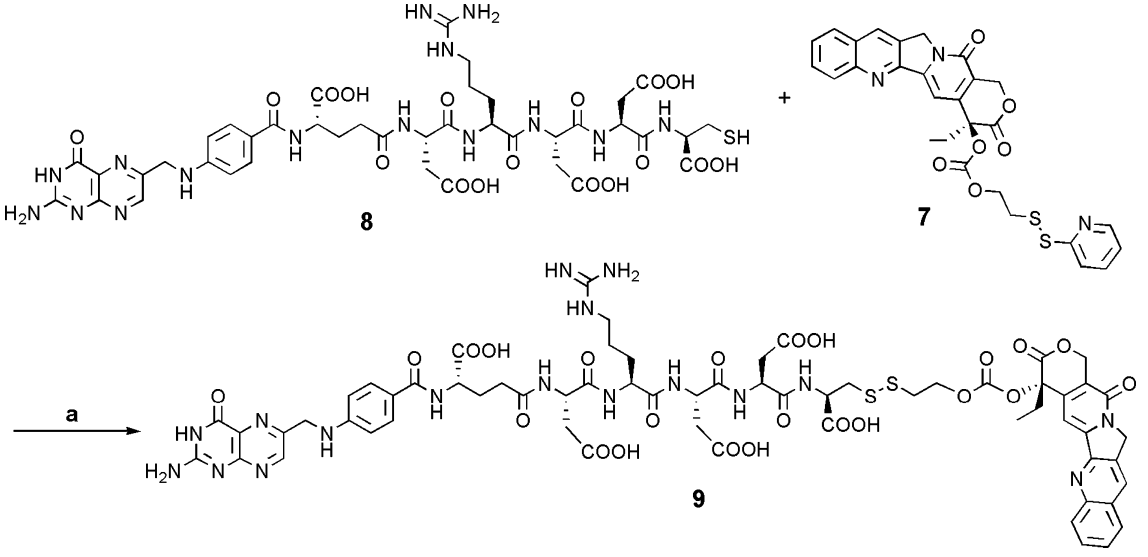
we hypothesize that linkage of the targeting moiety to the 20-OH of CPT will stabilize the lactone ring and thereby minimize systemic toxicity by ensuring full drug activity upon arrival at the tumor.

Following a procedure reported in the literature,[29] commercially available camptothecin (**1**) was treated with triphosgene (*caution: triphosgene should only be handled by trained personnel*) in the presence of DMAP to form the C20 chloroformate (**6**) of camptothecin. Addition of **5** to the chloroformate afforded pyridyldithioethyl carbonate camptothecin (**7**).[35] Coupling of the pyridyldithiol carbonate arm to camptothecin in this manner also allows for possible conjugation to other free thiols (via disulfide exchange), rendering this precursor amenable to linkage to a variety of targeting ligands (e.g., peptides, aptamers, antibodies, etc.). Additionally, reduction of the disulfide carbonate linker releases the parent camptothecin in unmodified form.

The folate-peptide construct with a terminal cysteine residue, Pte-γ-Glu-Asp-Arg-Asp-Asp-Cys (**8**), was synthesized on an H–Cys(Trt)-2-Cl–Trt resin using standard FMOC-protected amino acids and N^{10}–trifluoroacetylpteroic acid as previously described.[36,37]

Scheme 2 illustrates the conjugation of folate peptide (**8**) to **7** forming the completed folate-peptide-CPT prodrug (**9**).[38] For comparison, an analogue possessing an ester linkage (**10**) was synthesized using a modified literature procedure.[39] Briefly, 4-(2-(pyridinyldithio)-butanoic acid was used as the heterobifunctional bridging agent for esterification to the 20-OH of camptothecin. Conjugation of the ester form of pyridyl camptothecin to **8** was performed as described in Scheme 2.

Together with cytotoxicity analyses, studies were performed on folate-peptide-CPT to evaluate both the binding affinity and CPT release efficiency of the intact prodrug. In our experience, all three parameters are predictive of in vivo efficacy and can be used to guide further structural refinements. Relative binding affinity was assayed using methodology described by Westerhoff et al.,[40] where KB cells (a human cervical cancer cell line



Scheme 2. Synthesis of folate-peptide-CPT (carbonate linker). Reagents and conditions: (a) DMSO, rt, 6 h.

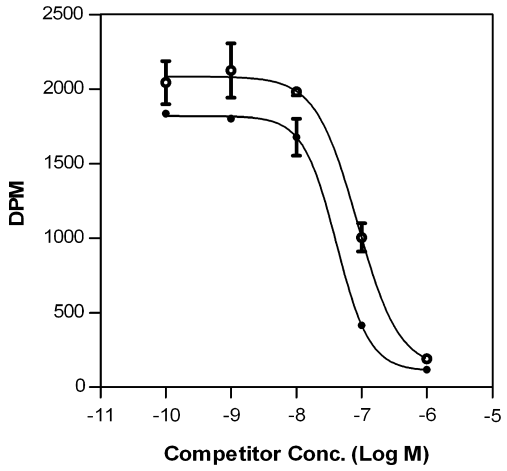
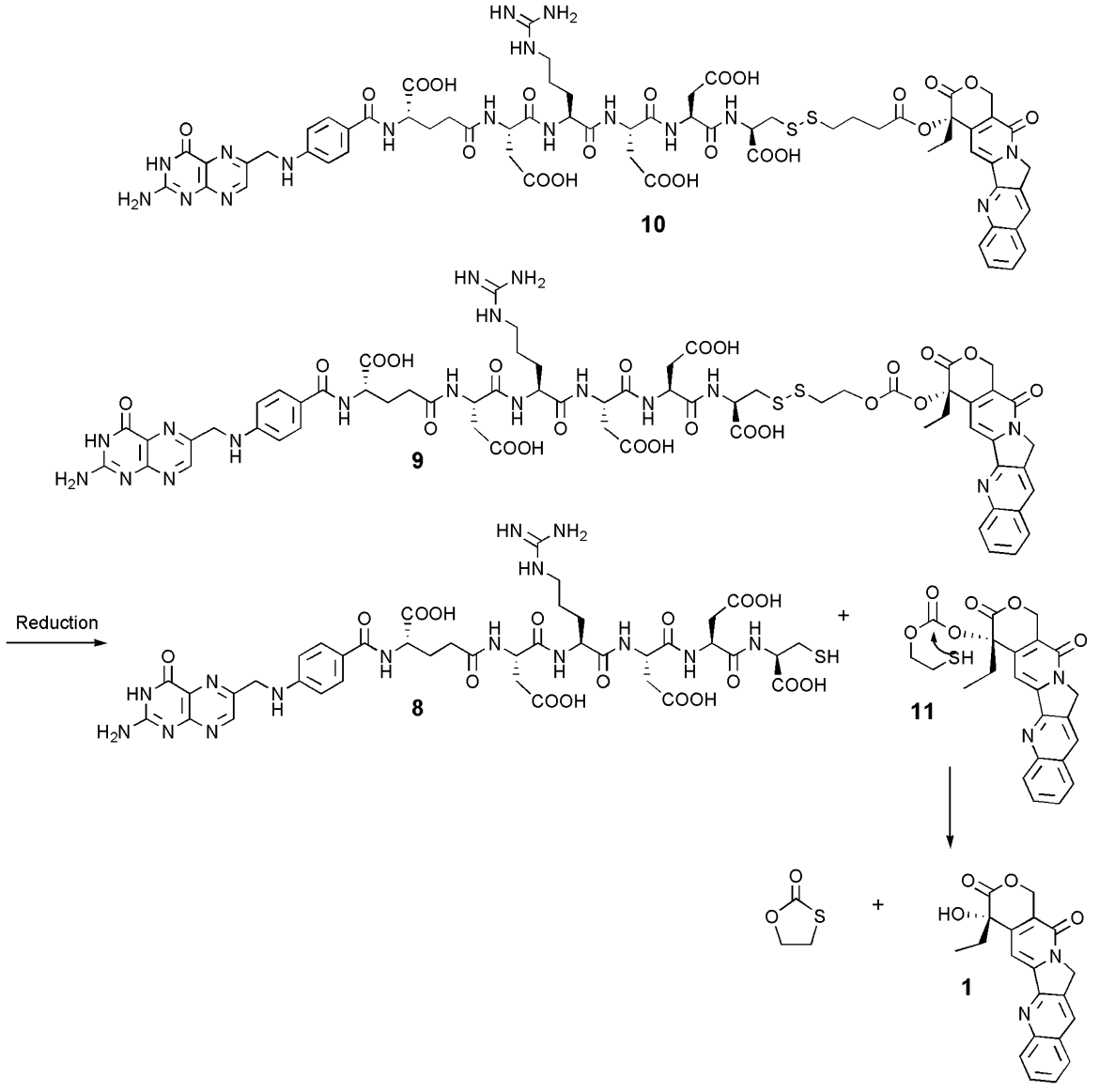


Figure 2. Relative folate receptor binding affinity of folate-peptide-CPT (**9**). Human KB cells were incubated for 30 min in the presence of 10 nM tritiated folic acid with increasing competitor concentrations. Open circles, folate-peptide-CPT; black circles, folic acid.

that over-expresses the folate receptor) were incubated in the presence of 10 nM ^{3}H folic acid plus increasing concentrations (0.1 nM–1 μM) of competitor (i.e., folate-peptide-CPT or non-radioactive folate). Relative affinity is defined as the molar ratio of competitor to folate required to block 50% of ^{3}H folic acid binding to KB cells. The relative affinity for folic acid, by definition, was set to 1. Thus, a relative affinity of 1 indicates a binding affinity equivalent to that of folic acid, whereas a lower value reflects lower affinity and a higher value indicates higher affinity. Analysis of the folate-peptide-CPT prodrug revealed a relative binding affinity of 0.44 (Fig. 2), demonstrating that attachment of the peptide spacer to camptothecin has little impact on FR binding.[41] To assess release of camptothecin (Scheme 3) from the folate peptide linker, folate-peptide-CPT was incubated in the presence of a 10-fold molar excess of the disulfide reducing agent, dithiothreitol, in 0.1 mM phosphate-buffered saline (pH 7.4) for 1 h. Based on HPLC analysis (Fig. 3), >80% conversion of the pro-



Scheme 3. Disulfide mediated release of camptothecin from the folate peptide carbonate linker.

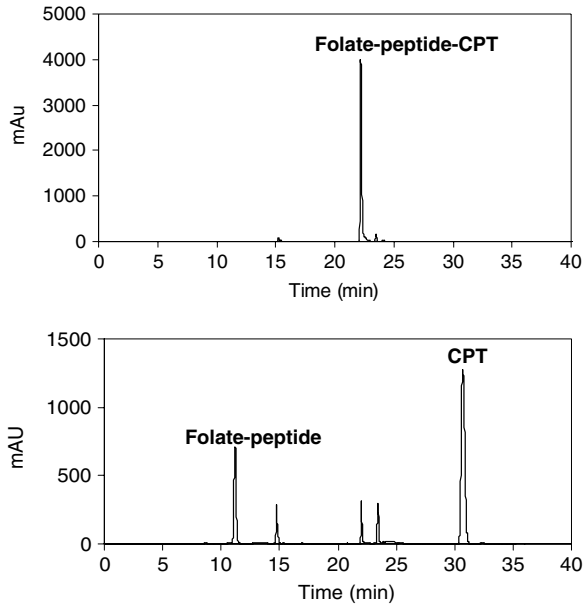


Figure 3. Release of CPT (**1**) from folate-peptide-CPT prodrug (**9**) by disulfide reduction. Folate-peptide-CPT was analyzed using RP-C18 HPLC (Abs = 280 nm) in the absence (top chromatogram) and presence (bottom chromatogram) of a 10-fold excess of dithiothreitol.

drug to free camptothecin occurred within the 1 h time frame.

In vitro cytotoxicity of folate-peptide-CPT was evaluated using a modified tritiated thymidine incorporation assay.[5] Confluent FR + KB cells in folate deficient medium were incubated for 1 h (pulsed) with increasing concentrations of folate-peptide-CPT in the presence or absence of 0.1 mM folic acid. After washing to remove unbound prodrug, the cells were incubated 72 h (chased) in fresh medium. This type of pulse-chase assay is considered more appropriate for evaluation of folate-targeted compounds than a standard 72 h incubation, since folate conjugates are commonly cleared from both the vasculature and interstitial spaces within a short time frame (<2 h).[5,41] Incubation of cells with folate conjugates in the presence of free folic acid further enables discernment of receptor mediated uptake from non-receptor mediated mechanisms. As shown in Figure 4, tritiated thymidine incorporation (a measure of DNA synthesis) decreased in a dose-dependent manner with increasing concentrations of folate-peptide-CPT ($IC_{50} \sim 10$ nM). Folate-peptide-CPT activity, furthermore, was quantitatively blocked in the presence of excess folic acid, demonstrating that prodrug uptake is FR mediated.

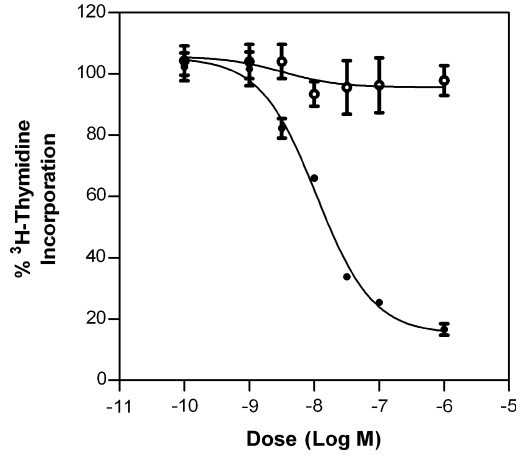


Figure 4. Dose-response of folate-peptide-CPT (**9**). Human KB cells were incubated with increasing concentrations of folate-peptide-CPT in the presence or absence of excess folic acid for 1 h. After a 72-h incubation in fresh media, activity was assessed using a tritiated thymidine incorporation assay. Open circles, folate-peptide-CPT plus 0.1 mM folic acid; black circles, folate-peptide-CPT. $IC_{50} \sim 10$ nM.

Recent data demonstrate that reduction of disulfide bonds in folate conjugates begins immediately following endocytosis and proceeds continuously until FR recycles to the cell surface.[42] Importantly, reduction of the disulfide bond in the carbonate prodrug **9** generates a thiol intermediate (**11**) that would be expected to cleave the carbonate bridge to CPT (Scheme 3), releasing unmodified camptothecin.[43] In contrast, generation of the same thiol intermediate in the analogous ester-bridged prodrug (**10**) would not be expected to facilitate rapid ester hydrolysis, resulting in less CPT release during prodrug endocytosis. Consistent with this anticipation, the cytotoxicity of the ester-linked prodrug was found to be much lower than the carbonate-linked prodrug (Fig. 5). The data also suggest that esterases capable of releasing CPT from the ester-linked prodrug are not abundant in FR + endosomes.

In summary, the synthesis of a novel folate peptide camptothecin prodrug, possessing a releasable disulfide carbonate linker, has been described. The disulfide carbonate-peptide spacer confers water solubility to the prodrug without compromising FR binding affinity and provides a release mechanism for rapid unloading of camptothecin within FR + cancer cells. Moreover, this synthetic strategy could prove useful for construction of additional camptothecin prodrugs linked to other targeting ligands.

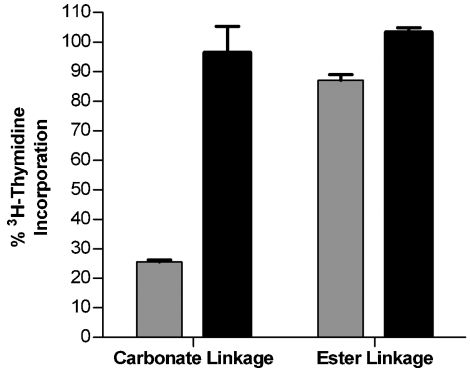


Figure 5. Comparison of carbonate versus ester drug release mechanism. Cells were incubated in 100 nM of each respective conjugate (carbonate linkage = compound **9**; ester linkage = compound **10**) in the presence or absence of an excess of folic acid for 1 h. After a 72-h incubation in fresh media, activity was assessed using the tritiated thymidine incorporation assay. Gray bars, 100 nM, respective conjugate. Black bars, 100 nM respective conjugate + 0.1 mM folic acid.

Acknowledgments

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References and notes

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34. Compound **5**: colorless viscous liquid, $R_f = 0.20$ (7:3 EtOAc/pet. ether) ^{1}H NMR (Bruker 500 MHz cryoprobe, DMSO-d_6) δ 2.92 (t, $J = 6.4$ Hz, 2H, S–CH_2); 3.62 (q, $J = 6.1$ Hz, 2H, O–CH_2); 5.00 (t, $J = 5.5$ Hz, 1H, OH); 7.24 (tt, $J = 5.74$, 1.4 Hz, 1H, Py–H); 7.83 (m, 2H, Py–H); 8.45 (d, $J = 4.9$ Hz, 1H, Py–H). ESI-MS = 187.98 $(M+H)^+$.
35. Compound **7**, Off-white solid, $R_f = 0.25$ (8:2 $CHCl_3$/acetone) ^{1}H NMR (Bruker 500 MHz cryoprobe, DMSO-d_6) δ 0.92 (t, $J = 7.4$ Hz, 3H, H19); 2.18 (m, 2H, H18); 3.14 (t, $J = 6.1$ Hz, 2H, S–CH_2); 4.32 (t, $J = 6.0$ Hz, 2H, O–CH_2); 5.30 (s, 2H, H5); 5.52 (d, $J = 3.5$ Hz, 2H, H17); 7.09 (s, 1H, H14); 7.15 (m, 1H, Py–H); 7.70 (m, 2H, Py–H); 7.77 (td, $J = 7.75$, 1.8 Hz, 1H, H10); 7.85 (td, $J = 8.0$, 1.0 Hz, 1H, H11); 8.13 (t, $J = 8.1$ Hz, 2H, H9 & H12); 8.39 (dt, $J = 4.8$, 0.7 Hz, 1H, Py–H); 8.69 (s, 1H, H7). ESI-MS = 562.05 $(M+H)^+$.
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37. Compound **8**: yellow solid, $R_t = 11.2$ min, RP-HPLC, Rigel (Stellar Phases), 5 μM C_{18}, 10×250 mm, 0.1 mM NH_4HCO_3, (pH 7) = A, ACN = B; 1 mL/min (1–99% B over 40 min, λ 280 and 360 nm. ^{1}H NMR (Bruker 500 MHz cryoprobe, DMSO-d_6/D_2O) δ 1.40 (m, 1H, Pep–H); 1.49 (m, 1H, Pep–H); 1.60 (m, 1H, Pep–H); 1.91 (m, 3H, Pep–H); 2.22 (m, 2H, Pep–H); 2.60–3.21 (ms, 8H); 4.18 (t, $J = 5.0$ Hz, 1H, Pep–αH); 4.23 (m, Pep–αH); 4.28 (dd, $J = 4.8$, 4.0 1H, Pep–αH); 4.34 (t, $J = 5.4$ Hz, 1H, Pep–αH); 4.47 (s, 2H, Ptc–H); 4.52 (t, $J = 6.8$ Hz, 1H, Pep–αH); 4.57 (t, $J = 6.4$ Hz, 1H, Pep–αH); 6.63 (d, $J = 8.7$ Hz, 2H, Ptc–Ar–H); 7.62 (d, $J = 8.6$ Hz, 2H, Ptc–Ar–H); 8.63 (s,1H, Ptc–Ar–H). ESI-MS = 1046.28 $(M+H)^+$.
38. Compound **9**: yellow solid, $R_t = 22.01$ min, RP-HPLC, Rigel (Stellar Phases), 5 μM C_{18}, 10×250 mm: 0.1 mM NH_4HCO_3, (pH 7) = A, ACN = B; 1 mL/min (1–99% B over 40 min): λ 280 and 360 nm. ^{1}H NMR (Bruker 500 MHz cryoprobe, DMSO-d_6/D_2O) δ 0.89 (t, $J = 7.4$ Hz, 3H, H19); 1.37 (m, 1H, Pep–H); 1.46 (m, 1H, Pep–H); 1.58 (m, 1H, Pep–H); 1.86 (m, 2H, Pep–H); 1.98 (m, 1H); 2.15 (m, 4H, H18 & Pep–H); 2.36–2.63 (ms, 7H, Pep–H); 2.94 (m, 3H, S–CH_2 & Pep–H); 3.02 (m, 1H, Pep–H); 3.14 (dd, $J = 7.6$, 5.3 Hz, 1H, Pep–H); 4.11 (t, $J = 5.8$ Hz, 1H, Pep–αH); 4.18 (t, $J = 6.5$ Hz, Pep–αH); 4.28 (m, 3H, O–CH_2 & Pep–αH); 4.34 (t, $J = 6.0$ Hz, 1H, Asp–αH); 4.46 (s, 2H, Ptc–H); 4.49 (m, 2H, Pep–αH); 5.30 (d, $J = 3.0$, 2H, H5); 5.50 (s, 2H, H17); 6.61 (d, $J = 8.7$ Hz, 2H, Ptc–Ar–H); 7.13 (s, 1H, H14); 7.58 (d, $J = 8.6$ Hz, 2H, Ptc–Ar–H); 7.70 (t, $J = 7.6$ Hz, 1H, H10); 7.86 (t, $J = 7.6$ Hz, 1H, H11); 8.10 (d, $J = 8.2$ Hz, 1H, H9); 8.18 (d, $J = 8.5$ Hz, 1H, H12); 8.62 (s,1H, Ptc–Ar–H); 8.68 (s,1H, H7). ESI-MS = 1496.17 $(M+H)^+$.
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