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ORIGINAL ARTICLE

Synthesis of camptothecin–amino acid carbamate linkers

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Abstract A more convenient and facile approach for the synthesis and production of camptothecin–amino acids carbamate linkers, that can be used in the synthesis of bioconjugate peptides JF-10-81, JF-10-71, and other peptide analogs designed to target somatostatin receptors has been described.

Keywords Camptothecin · Somatostatin · Bioconjugates · Amino acids · Linkers

Introduction

Prodrugs are inactive compounds that become site-specific and active pharmacological agents once metabolized in vivo (Kratz et al. 2008). Through the use of these agents, it is possible to enhance the therapeutic efficacy against diseases such as carcinomas. A cleavable linker moiety is a vital component in prodrug design and researchers have explored various chemistry design strategies. Despite the use of several different chemical functionalities, most linker moieties release mechanisms have been categorized as either elimination-based release or cyclization-based release (Papot et al. 2002; Tranoy-Opalinski et al. 2008). The cyclization-based method has received increased attention in recent years and can proceed via three types of intramolecular cyclization that result in the release of active therapeutic agents. Optimization of the linker moiety is paramount in prodrug design as it regulates stability, release rates, and enhanced drug potency (Fuselier et al. 2003; Gomes et al. 2007; Papot et al. 2002; Shan et al. 1997; Tranoy-Opalinski et al. 2008; Vigroux et al. 1995).

Herein we discuss the synthesis of two camptothecin (CPT) built-in nucleophilic-assisted releasing (BINAR) amino acid carbamate linkers and their ready attachment to a model somatostatin peptide analog selective for the type 2 somatostatin receptor (SST2). The camptothecin–somatostatin peptide hormone conjugates (CPT-SSA) retain high binding affinity to sst_2, a somatostatin receptor subtype that is over-expressed in many cancerous tumors such as those of the breast, lung, and prostate. The CPT-SSA conjugates described here have been shown to effectively target tumor cells due to the introduction of the BINAR carbamate linker which is capable of intracellularly releasing camptothecin selectively to tumor cells via a "two-step" cyclization–elimination mechanism. The linker stability, release rates, and drug potency of JF-10-71 and JF-10-81 have been discussed previously (Bezerra et al. 2005; Fuselier et al. 2003; Sun et al. 2004; Sun and Coy 2008). The on-resin synthesis of these CPT-SSAs has also been described (Fuselier et al. 2003), however, low yields have been observed using the previously reported synthetic route. The focus of this publication is to elaborate on a convenient and facile route for synthesizing mass quantities of one of these bioconjugates for use in clinical development for the treatment of age-related macular degeneration.

Materials and methods

^{1}H NMR spectra were recorded using a 500 MHz Bruker spectrometer. $CDCl_3$, D_2O, and DMSO-d_6 were used as

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solvents. The chemical shifts are presented in ppm in the δ scale relative to internal HMDS. The melting points of substances were determined on an electrothermal melting point apparatus. All solvents were purified by standard procedures.

2-(2-(*tert*-Butoxycarbonyl(methyl)amino)ethylamino) acetic acid (**8**)

Tert-Butyl 2-aminoethyl(methyl)carbamate (**4**) (5 g, 28.7 mmol) in water (10 mL) was added dropwise to glyoxylic acid monohydrate (2.10 g, 22.9 mmol) in water ($\sim$25 mL) at 35°C and the reaction mixture was allowed to stir for 18 h. During this time, the reaction was monitored by TLC (BAW, 4:1:1) as the Schiff base formed to yield 6, which was not isolated. To this solution, an additional 100 mL of water was added and allowed to cool to room temperature. The solution was then reduced using 5% Pd/C (910 mg; 1 mol/20 g) purging with excess hydrogen gas through a mercury bubbler for 24 h. Pd/C was filtered and the resulting yellow solution was concentrated, re-suspended in ethyl ether, and filtered to yield 8, white powder, yield (2.88 g, 43%). m.p. = 183–185°C. ^{1}H NMR (500 MHz, D_2O): δ (ppm) 1.4 (w.s, 9H, –C(CH_3)$_3$), 2.8(s, 3H, NH–CH_3), 3.2(s, 2H, –CH_2–COOH), 3.5(w.s, 4H, –NH–CH_2–CH_2–NCH_3Boc); ESI-MS *m*/*z*: 233.20 ([M + H]$^+$, calcd 232.27).

2-(2-(*tert*-Butoxycarbonyl)amino)ethylamino) acetic acid (**9**)

Tert-Butyl(2-aminoethyl)carbamate (**5**) (5 g, 31.2 mmol) in water (10 mL) was added dropwise to glyoxylic acid monohydrate (2.30 g, 24.96 mmol) in water ($\sim$25 mL) at 35°C and the reaction mixture was allowed to stir (18 h). During this time, the reaction was monitored by TLC (BAW, 4:1:1) as the Schiff base formed to yield 7, which was not isolated. To this solution, an additional 100 mL of water was added and allowed to cool to room temperature. The solution was then reduced using 5% Pd/C (910 mg; 1 mol/20 g) purging with excess hydrogen gas through a mercury bubbler for 24 h. Pd/C was filtered and the resulting yellow solution was concentrated, re-suspended in ethyl ether, and filtered then washed with ethyl acetate to yield 9, white powder. Yield (2.53 g, 46.5%). m.p. = 211–213°C ^{1}H NMR (500 MHz, D_2O): δ (ppm) 1.4(w.s, 9H, –C(CH_3)$_3$), 3.2(t, 2H, –NH–CH_2–), 3.4(t, 2H, –CH_2–NHBoc), 3.6(s, 2H, –NH–CH_2–COOH); ESI–MS *m*/*z*: 219.13 ([M + H]$^+$, 241.11 [M + Na]$^+$, calcd 218.25).

Ethyl 2-(2-(*tert*-butoxycarbonyl(methyl)amino) ethylamino)acetate (**10**)

Ethyl bromoacetate (554 mL, 5 mmol), in 5 mL of anhydrous MeCN, was added dropwise to 4 (871 mg, 5 mmol) in 30 mL of anhydrous MeCN at 0°C and then allowed to warm to room temperature. The reaction was monitored by TLC (95:5 EtOAc:MeOH) for 5 h. After completion, the solution was concentrated under reduced pressure and the resulting mixture was dissolved in water and extracted with ethyl acetate (3×). The organic layer was dried over $MgSO_4$ then concentrated under reduced pressure. The crude product was purified via silica gel column chromatography (95:5 EtOAc:MeOH) to yield (**10**) as a clear oil (730 mg, 71% yield).). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.2 (m, 2H, CH_3CH_2), 3.4 (t, 2H, *N*(Me)–CH_2), 3.3 (m, 2H, $NHCH_2$), 2.8(s, 3H, NCH_3), 2.7(s, 2H, NH–CH_2–COOEt), 1.4 (m, 9H, C–(CH_3)$_3$), 1.2 (t, 3H, CH_2CH_3); ESI–MS *m*/*z*: 261.18 ([M + H]$^+$, calcd 260.33).

2-(2-(*tert*-Butoxycarbonyl(methyl)amino)ethylamino) acetic acid sodium salt (**11**)

Ethyl 2-(2-(*tert*-butoxycarbonyl(methyl)amino)ethylamino) acetate (**10**) (730 mg, 2.80 mmol) was dissolved in 40 mL of 3 N sodium hydroxide and ethanol (1:1) and allowed to react at room temperature overnight. The solvent was then removed under reduced pressure to yield crude hydrolyzed product (turbid yellow oil). The crude product was purified via silica gel column chromatography (100% MeOH) and triturated in Et_2O to yield 11 as a white solid (quantitative yield). m.p.: >300°C (Na^+ salt); ^{1}H NMR (500 MHz, D_2O):δ (ppm) 3.4 (s, 2H, NH–CH_2–COOH), 3.2 (t, 2H, *N*(Me)–CH_2), 2.8 (s, 3H, NCH_3), 2.7 (m, 2H, $NHCH_2$), 1.4 (m, 9H, C–(CH_3)$_3$); ESI–MS *m*/*z*: 255.13 ([M + Na]$^+$, calcd 232.28)

(4*S*)-4-Ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-4-yl-trichloromethyl carbonate (**12**)

DMAP (3.52 g, 28.8 mmol) dissolved in DCM (20 mL), freshly distilled from CaH_2, was added dropwise over 10 min to a suspension of camptothecin (3.16 g, 9.08 mmol) and triphosgene (0.99 g, 3.33 mmol) in anhydrous DCM ($\sim$200 mL) under a stream of nitrogen. The yellow suspension became a red/rose homogenous solution and was allowed to react for 1 h to yield 12 which was not isolated.

(4*R*)-4-Ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′: 6,7]indolizino[1,2-b]quinolin-4-yl 4 nitrophenyl carbonate (**13**)

Camptothecin (5.00 g, 14.37 mmol) and PNP-chloroformate (8.69 g, 43.11 mmol) were dissolved in freshly distilled DCM (450 mL) under nitrogen atmosphere at 0°C. DMAP (10.53 g, 86.22 mmol), in 30 mL of anhydrous

DCM, was added dropwise to the chilled suspension. Upon complete addition of DMAP, the yellow-turbid suspension was then a brown suspension and the reaction mixture was allowed to react for 3 h at room temperature. The EtOAc:MeOH). After completion, the reaction mixture was filtered and the golden colored filtrate was washed with 5% $KHSO_4$ (3×, 200 mL). The organic layers were combined dried over $MgSO_4$ then concentrated under reduced pressure to half the volume and precipitated with Et_2O. The yellow solid product was filtered and dried to yield CPT-PNP carbonate 13 (5 g, 68% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm); 1.1 (t, 3H, CH_2–CH_3), 2.2–2.3 (m, 2H, CH_2–CH_3), 5.3 (d.d, 2H, –NCH_2), 5.4 (d, 1H, –CH–O–), 5.7 (d, 1H, –CH–O–) 7.2 (s, 1H, –C=CH), 7.4–8.5 (m, 9H, Ar–H); ESI-MS *m*/*z*: 514.13 ($[M + H]^+$, calcd 513.46).

Trimethylsilyl-2-(2-(Boc-methylamino)ethyl) (trimethylsilyl)amino) acetate (**14**)

2-(2-(*tert*-Butoxycarbonyl(methyl)amino)ethylamino) acetic acid (**8**) (2.14 g, 9.20 mmol) was suspended in anhydrous DCM (30 mL), freshly distilled from CaH_2, and DIPEA (2.37 g, 18.4 mmol) was added at once under nitrogen flow. TMS-Cl (1.99 g, 18.4 mmol) was slowly added to the suspension via syringe. Once the solution became homogeneous, it was refluxed for 1 h to yield 14, which was not isolated.

Trimethylsilyl-2-(2-(Boc-amino)ethyl)(trimethylsilyl) amino) acetate (**15**)

2-(2-(*tert*-Butoxycarbonyl)amino)ethylamino) acetic acid (9) (2.02 g, 9.25 mmol) was suspended in anhydrous DCM (30 mL), freshly distilled from CaH_2, and DIPEA (2.38 g, 18.5 mmol) was added at once under nitrogen flow. TMS-Cl (1.99 g, 18.5 mmol) was slowly added to the suspension via syringe. Once the solution became homogeneous, it was refluxed for 1 h to yield 15, which was not isolated.

N-{2-[(*tert*-Butoxycarbonyl)(methyl)amino]ethyl}-*N*-({[(4*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-4-yl]oxy}carbonyl)glycine (**16**)

The solution containing (**14**) was slowly added to the solution containing (**12**) via cannula under nitrogen atmosphere and allowed to react while stirring overnight at room temperature. After overnight mixing, the reaction mixture was filtered and washed several times with 5% $KHSO_4$. The organic layers were dried with Na_2SO_4 then concentrated under reduced pressure. The crude product was purified on silica gel using stepwise solvent gradients. The crude material was dissolved in DCM, washed with several column volumes of DCM, followed by elution of free camptothecin using 5% MeOH in DCM. 16 was eluted with 10% MeOH in DCM and concentrated under reduced pressure to yield a light yellow solid. Yield (3.07 g, 56%).^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm); 0.9 (t, 3H, CH_2–CH_3), 1.2–1.4 (b.s., 9H, –$C(CH_3)_3$), 2.0–2.2 (m, 2H, CH_2–CH_3), 2.7 (s, 3H, N–CH_3), 2.9–3.6 (m, 6H, –CH_2–CH_2–*N*(Me)Boc, –CH_2–COOH) 5.3 (d.d, 2H, –NCH_2–CPT fragment), 5.5 (d, 2H, –CH_2–O–CPT fragment), 7.15 (s, 1H, –C=CH), 7.7–8.7 (m, 5H, Ar–H); ESI-MS *m*/*z*: 607.24 ($M + H^+$, 1213.47 dimer M–M, calcd 606.6).

N-{2-[(*tert*-Butoxycarbonyl)(methyl)amino]ethyl}-*N*-({[(4*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-4-yl]oxy}carbonyl)glycine (**16**)

Alternatively compound 16 was prepared from CPT-PNP carbonate 13 in anhydrous DCM. Compound **8** (2.40 g, 10 mmol) was suspended in anhydrous DCM (30 mL), freshly distilled from CaH_2, and DIPEA (2.58 g, 20.0 mmol) was added at once under nitrogen flow. TMS-Cl (2.17 g, 20.0 mmol) was slowly added to the suspension via syringe. Once the solution became homogeneous, it was refluxed for 1 h to yield **14**, which was not isolated. The solution containing 14 was chilled to 0°C and CPT-PNP carbonate, **13**, dissolved in freshly distilled DCM (~50 mL), was added via cannula under nitrogen atmosphere and stirred overnight at room temperature. After overnight mixing, the reaction mixture was filtered and washed several times with 5% $KHSO_4$, where a small amount of 16 precipitated and was isolated. The organic layers were dried with Na_2SO_4 then concentrated under reduced pressure. The crude product was purified via silica gel column chromatography (95:5 DCM:MeOH) to give **16** as a yellow solid. The yellow solid was triturated with ethyl ether and filtered to yield a light yellow powder (3.5 g, 58% yield). ESI–MS *m*/*z*: 607.24 ($[M + H]^+$, calcd 606.23). ^{1}H NMR (500 MHz, DMSO-d_6): same as previously described spectrum.

N-{2-[(*tert*-Butoxycarbonyl)amino]ethyl}-*N*-({[(4*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinolin-4-yl]oxy}carbonyl)glycine (CPT-BINAR glycine) (**17**)

The solution containing **15** was slowly added to the solution containing **12** via cannula under nitrogen atmosphere and allowed to react while stirring overnight at room temperature. After overnight mixing, the reaction mixture was filtered and washed several times with 5% $KHSO_4$. The organic layers were dried with Na_2SO_4 then concentrated under reduced pressure. The crude product was

purified on silica gel using stepwise solvent gradients. The crude was dissolved in DCM, washed with several column volumes of DCM, followed by elution of free camptothecin using 5% MeOH in DCM. **17** was eluted with 10% MeOH in DCM and concentrated under reduced pressure to yield a light yellow solid. Yield (0.25 g, 4.64%). MALDI *m/z*: 616.9 ([M + Na] calcd 592.23). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm); 0.9 (t, 3H, CH_2–CH_3), 1.15–1.4 [b.s., 9H, –C(CH_3)$_3$], 2.0–2.15 (m, 2H, CH_2–CH_3), 2.9–3.6 (m, 6H, –CH_2–CH_2–*N*(Me)Boc, –CH_2–COOH) 5.3 (d.d, 2H, –NCH_2–CPT fragment), 5.5 (d, 2H, –CH_2–O–CPT fragment), 7.15 (s, 1H, –C=CH), 7.6–8.7 (m, 5H, Ar–H)

Solid-phase synthesis of CPT-BINAR-D-Ser-Nle-D-Lys-D-Tyr-D-Ser-[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-NH_2

Automated solid-phase peptide synthesis was carried out on a CS136 Peptide Synthesizer (CS Bio, Inc., CA) using FMOC amino acids with appropriate side-chain protection. Rink amide methylbenzhydrylamine resin (0.25 mmol) was added to a reaction vessel and allowed to swell in *N,N*-dimethylformamide (DMF) for approximately 1 h. The resin was filtered and an excess of 20% piperidine in DMF was added and mixed for 2 min to remove the resin FMOC group. The resin was filtered and again washed with an excess amount of 20% piperidine for 20 min. After deprotection, the resin was washed with excess DMF, then FMOC-Thr(tBut) (3 M eq.), diisopropylcarbodiimide (DIC, 0.3 M), and HOBt (6 M eq) in DMF were added to the resin. The resin was mixed for 1 h then washed four times with DMF or NMP. Kaiser tests were conducted to test qualitative coupling efficiencies. If necessary, a double coupling of the appropriate amino acid was performed using 3 equivalents of TBTU and 6 equivalents of *N,N′*-diisopropylethylamine (DIPEA) dissolved in DMF. The remaining amino acids, FMOC-*O*-*t*-butyl-L-threonine, FMOC-*S*-trityl-L-cysteine, FMOC-*O*-*t*-butyl-L-threonine, N^{α}-FMOC-N^{ε}-Boc-L-lysine, N^{α}-FMOC-*N*-Boc-D-tryptophan, FMOC-L-phenylalanine, FMOC-S-trityl-L-cysteine, FMOC-*O*-*t*-butyl-D-serine, FMOC-*O*-*t*-butyl-D-tyrosine, FMOC-norleucine, FMOC-*O*-*t*-butyl-D-serine, in their respective order, were *N*-FMOC deprotected and coupled to the peptidyl resin using DIC/HOBt coupling procedure. Following the final FMOC deprotection procedure, BINAR amino acid linker (**16** or **17**) was coupled to the resin using DIC/HOBt coupling procedure and the resin was washed with MeOH and dried. After complete assembly of peptide chain, the peptide was cleaved from the solid support and side-chain deprotection was performed using a cleavage cocktail of TFA:H_2O:EDT:TIPS (84: 2.5:2.5:1) while shaking for 2 h then filtered. The resin was then washed several times with TFA and the combined filtrate was reduced almost to dryness. The cleaved/deprotected CPT-BINAR-peptide was precipitated by dropwise addition of the filtrate in excess cold ether. The flocculent precipitate was centrifuged and washed several times with cold ether to give a white powder. The peptide chain was then cyclized by dissolving in a large volume of 60% acetic acid and adding a few drops of I_2 in MeOH until a slight permanent brown coloration resulted. A pinch of ascorbic acid was added to the solution following cyclization to scavenge excess I_2. The solvent was then reduced to a volume of 10–15 mL and was subjected to a preparative RP-HPLC column containing C-18 bonded silica gel (Dynamax-300 A, 8 μm, 21.4 × 250 mm). Linear gradients 0.1% TFA in H_2O (v/v) (Buffer A) and 0.1% TFA in 80% MeCN (v/v) (Buffer B) were used in all preparative RP-HPLC. The separation was monitored at 280 nm with an elution of buffer B: 20% B to 50% B over 80 min; flow rate of 20 mL/min. The fractions containing ≥95% pure (analytical HPLC) product were pooled, concentrated in vacuo and lyophilized. The peptide was obtained as a fluffy light yellow powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed by analytical RP-HPLC. Analytical RP-HPLC results were recorded using a Vydac C-18 support (4.6 × 250 mm, 5 μm, 300 Å pore size; Liquid Separations Group) with linear gradients 0.1% TFA in H_2O (v/v) (Buffer A) and 0.1% TFA in 80% MeCN (v/v). Column eluant, 20% B to 50% B over 30 min; flow rate of 1.5 mL/min, was monitored at 215 nm. The retention time and purity of the peptide was assessed by the Rainin Dynamax HPLC Method Manager. Peptide was characterized by MALDI-MS.

Bioconjugate peptide JF-10-81 Synthesized using BINAR amino acid linker **16**. Peptide was characterized by MALDI-MS *m/z*: 1824.93 (calcd 1824.04).

Bioconjugate peptide JF-10-71 Synthesized using BINAR amino acid linker **17**. Peptide was characterized by MALDI-MS *m/z*: 1812.31 (calcd 1810.02).

Discussion

The previously reported solid-phase synthesis of CPT-peptide conjugates JF-10-71 and JF-10-81 suffered from inefficient yields and poor scalability (Fuselier et al. 2003). Current literature describes several methods for synthesizing various CPT-conjugates (Burke et al. 2009; Leu et al. 1999, Ohwada et al. 2009) but the most widely used involve conversion of the C(20)-OH hydroxyl group of CPT to either a chloroformate derivative or a carbonate derivative followed by reaction with various nucleophiles and peptide derivatives (Chen and Gabathuler 2004; de Groot et al. 2002; Henne et al. 2006; Lee et al. 2010; Pessah et al. 2004; Zhao et al. 2000). In this vein, we devised a convenient

method of synthesizing CPT-BINAR amino acid linkers and their peptide-conjugated derivatives.

The appropriate diamines, *N*-methylethylenediamine and ethylenediamine, were chosen as optimal starting materials because both were commercially available. *tert*-Butyl-2-aminoethyl(methyl)carbamate, **4**, was synthesized in 93% yield using previously described protocol (Martins et al. 2001; Saari et al. 1990) and was further purified via vacuum distillation. *tert*-Butyl-2-aminoethylcarbamate, **5**, was also synthesized as previously described (McKenna et al. 2009) whereupon acylation of the diamine was achieved with di-*tert*-butyl-dicarbonate (BOC, 1/6 equiv) in dichloromethane (DCM). BOC was chosen for amino temporary protection because it was compatible with subsequent solid-phase side-chain removal chemistry.

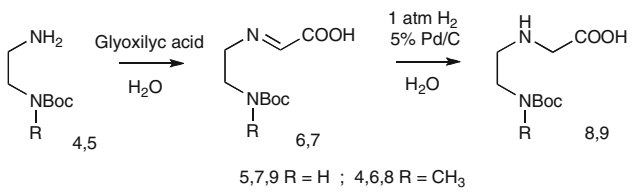
Two methods of synthesizing BINAR amino acid linkers were developed. The first involved the synthesis of amino acids **8** and **9** (Scheme 1), where diamines **4** and **5** were reacted with glyoxylic acid in water to form the respective Schiff bases **6** and **7**. Schiff bases **6** and **7** were obtained at constant mixing and heating at 35°C. Catalytic hydrogenation of the imines using 5% Pd/C (1 mol/20 g) yielded BINAR amino acid gylcinates **8** and **9** in moderate yields. ^{1}H NMR signals at 3.2 ppm (**8**) and 3.6 ppm (**9**) confirmed the presence of the two hydrogens of the glycine fragment RNH–CH_2-COOH of **8** and **9** compared to precursors **4** and **5**, respectively.

The second approach involved the synthesis of BINAR amino acid from commercially available ester precursors. Amination of ethyl bromoacetate with **4** yielded the desired *N*-Me-*N*-BOC-BINAR glycinate, **10**. Hydrolysis of the ethyl ester with NaOH/MeOH afforded the amino acid sodium salt of **8** (glycinate compound **11**) in quantitative yields but was not used in the synthesis of CPT-BINAR conjugates. The reaction between bromoacetic ethyl ester and diamine could be carried out under mild conditions at 0°C.

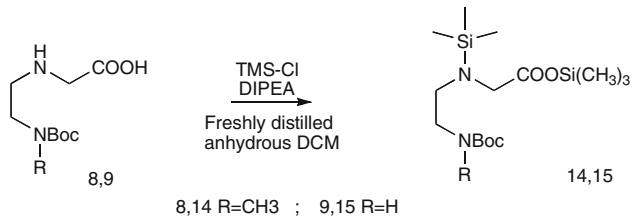
The allyl ester and benzyl ester derivatives of the BINAR linker were synthesized but were found not to be beneficial in the synthesis of CPT-BINAR conjugates. Bromoacetyl bromide was reacted with allyl alcohol (1 equiv) to form allyl bromoacetate which was amidated with **4** to yield the BINAR amino acid linker with *O*-allyl protection. This particular derivative was previously synthesized and conjugated to CPT via solid-phase methodologies (Fuselier et al. 2003). Solution phase synthetic strategy resulted in 47% yield of the desired compound, however, upon conjugation to CPT, deprotection of the allyl group was problematic. Several deprotection methods were used but yields of the desired bioconjugate, **JF-10-81**, were low (<4%). Benzyl bromoacetate was also amidated with **4** to yield the benzyl protected glycinate ester. Efficient reduction of the benzyl ester to afford the free acid **8** using Pd/C or Raney Ni and excess hydrogen gas was unachievable as reduced pressure was necessary for deprotection. The *tert*-butyl ester was not synthesized because, to maintain proper functionality and selectivity, the amino acid linker should be orthogonal.

CPT conjugation is more easily achieved through the C(20) hydroxyl group and CPT retains its antitumor activity via this linkage site. Direct acylation of the C(20) hydroxyl group with linker moieties, peptide spacers, and other small molecules is extremely problematic. Previously performed methods such as Schotten–Baumann and formation of carbamoyl chloride intermediates followed by direct acylation of BINAR glycinate free acids were unsuccessful in yielding desired bioconjugates. To circumvent the inefficacious results, efficient conjugation of CPT to yield stable carbamate linked bioconjugates **16** and **17** was achieved through the formation of carbonate precursor **13**, chloroformate precursor **12**, and *O*-*N*-bis-trimethylsilyl intermediates **14** and **15**.

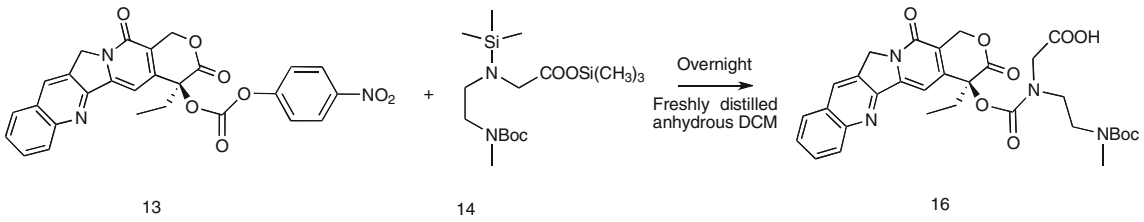
Acylation of compounds **8** and **9** was achieved using a modified protocol first introduced by Bolin et al. (1989). *O*-*N*-bis-TMS intermediates **14** and **15** (Scheme 2) were formed in situ. CPT-PNP-carbonate **13** was synthesized from the reaction of PNP-chloroformate (3 equiv), DMAP (6 equiv), and CPT in anhydrous DCM at 0°C. Amidation of carbonate **13** with TMS-protected intermediate **14** afforded bioconjugate **16**, 58% yield (Scheme 3). Additionally, CPT was reacted with triphosgene (1/3 equiv) and DMAP (3.2 equiv) in anhydrous DCM to form CPT-chloroformate **12** in situ. The CPT-chloroformate was then amidated with intermediates **14** and **15** to yield bioconjugates **16** and **17**, 56 and 4.65% yield, respectively (Scheme 4). It is helpful to note that the CPT-chloroformate **12**, CPT-carbonate **13**, and *O*-*N*-bis-trimethylsilyl intermediate of the BINAR glycinates **14** and **15,** were synthesized simultaneously.



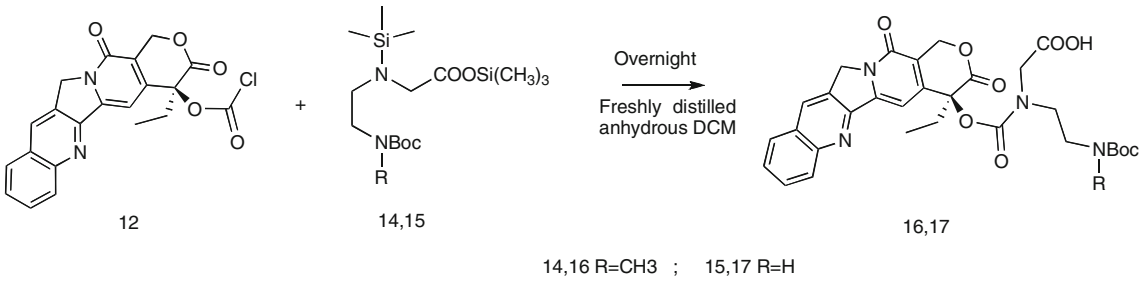
Scheme 1 Synthesis of BINAR-glycinate linkers



Scheme 2 Synthesis of O-Nbis-TMS protected BINAR-glycinate linkers



Scheme 3 Synthesis of CPT-BINAR bioconjugate from CPT-PNP carbonate precursor



Scheme 4 Synthesis of CPT-BINAR bioconjugates from CPT chloroformate precursor

The tumor recognition agent plays an important role in prodrug therapy because it mediates cell specificity and efficient delivery of the therapeutic agent to targeted cells. Immunoglobulins (antibodies) have been widely used as tumor recognition agents; however, limited success has been reached using these agents due to drawbacks associated with linker stability, cell selectivity, and drug potency (Chari 1998; Ducry and Stump 2010; Granett 2001). The use of peptides/peptide receptors as tumor recognition agents has been employed because it might be advantageous due to their in vivo targeting of tumor cells. It has been shown that peptide receptors are often over-expressed in tumor cells and display high binding affinities with peptide analogs. This enhanced selectivity should minimize cytotoxic side-effects in current chemotherapeutics (damage to neighboring healthy cells) whilst still inhibiting tumor growth (Czerwinski et al. 1998; Moody et al. 2005; Reubi 2003; Sun and Coy 2008).

Bioconjugates **16** and **17** were successfully coupled to a sst_2 specific somatostatin analog using solid-phase peptide chemistry and have shown promise as prodrug candidates. In vitro and in vivo studies have shown that the desired CPT-bioconjugates (JF-10-81 and JF-10-71) are stable in circulation and are potent cytotoxic agents in various cell lines (Fuselier et al. 2003; Sun et al. 2004). These newly designed peptide conjugates also exhibited low toxicity and were effective in an animal model of age-related macular degeneration, a degenerative disease of the retina (Bezerra et al. 2005).

In conclusion, an improved method for synthesizing glycinate-BINAR amino acids has been devised. Although several diamino linkers have been reported (Kumar et al. 2007; Papot et al. 2002; Pessah et al. 2004; Tranoy-Opalinski et al. 2008), they primarily serve as carbamate linkers and/or conjugate spacers. The presented amino acid linker design has a wide range of important applications. They can be incorporated not only as linker moieties, but could also be used as unusual *N*-amino acids in proteomic research. This is more advantageous because they are synthetically orthogonal and are compatible with solid-phase peptide synthesis. They also have similar chemical functionality (activation/coupling mechanisms) as standard *N*-protected-glycine amino acids. Compounds **8** and **9** offer a more facile and convenient approach for attaching therapeutic agents to various chemical/biochemical moieties, more specifically, peptide carriers via carbamate linkers. Additionally, monomer and polymeric derivatives of this amino acid linker have been incorporated in peptide nucleic acid (PNA) backbone design (Neilson 1999; Viirre and Hudson 2002).

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Conflict of interest M.A. Etienne, M. Kostochka, J.A. Fuselier, D. H. Coy, and Tulane University have proprietary rights to amino acid carbamate linkers and somatostatin conjugates described herein.

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