Site-specific traceless coupling of potent cytotoxic drugs to recombinant antibodies for pharmacodelivery.

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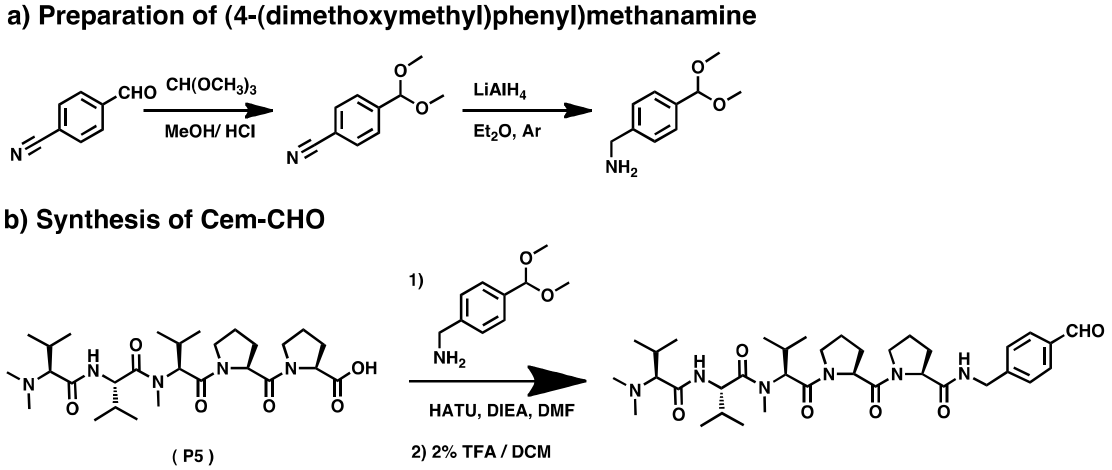
1. Synthesis of Cem-CHO

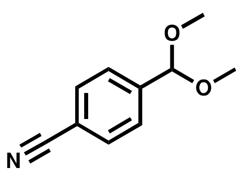
Material and methods: analytical and preparative high performance liquid chromatography (HPLC) were performed on an Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4 μ, Polar-RP 150*10 mm at a flow rate of 4 mL/min with linear gradients of solvents A and B (A= Millipore water with 0.1% TFA, B= Acetonitrile; 3 min. isocratic flow at initial conditions, followed by the gradient described in each procedure).


a) Preparation of (4-(dimethoxymethyl)phenyl)methanamine

b) Synthesis of Cem-CHO

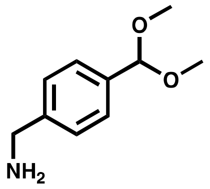
Figure SI1. Synthesis of cemadotin aldehyde (Cem-CHO)

4-(dimethoxymethyl)benzonitrile



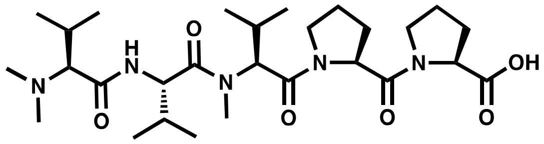
4-Formylbenzonitrile was converted into the corresponding dimethylacetal as previously described.[1] Briefly, in a three-neck flask 4-formylbenzonitrile (3 g, 22.88 mmol), MeOH (40 mL), trymethylorthoformate (15.2 mL, 137 mmol, 6 equiv.) and HCl (0.21 mL, 6.68 mmol, 0.3 equiv.) were added and the mixture heated for 16 h at 45 °C. The reaction was then evaporated, diluted with MeOH (20 mL) and hexanes. The organic layers were washed with Na_2CO_3 sat. sol., combined, dried over Na_2SO_4 and evaporated. The desired product was isolated as a yellowish solid (3.90 g, 21.9 mmol, 96% yield) and was used for the next synthetic step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.30 (s, 6H), 5.40 (s, 1H), 7.54-7.66 (m, 4H, AA’BB’); ESI-MS: calcd for $C_{10}H_{12}NO_2$ ($[M+H]^+$) *m/z* 178.20, found 177.80.

(4-(dimethoxymethyl)phenyl)methanamine



4-(dimethoxymethyl)benzonitrile was converted to the amine derivative as previously described.[1] Briefly, to a $LiAlH_4$ suspention in dry Et_2O (2 mL) in a 10 mL Schlenk flask, a Et_2O (2 mL) solution of 4-(dimethoxymethyl)benzonitrile (3.9 g, 21.95 mmol) was added dropwise at 0 °C. The reaction was then allowed to warm up to room temperature, and stirred for 2 extra hours. The reaction was quenched on ice with 1.7 mL H_2O, 1.7 mL 15% NaOH, and 5.1 mL H_2O. After the vigorous reaction had stopped, the suspension was filtered (celite plug), the solid was washed with Et_2O, and the combined organic layers were dried over Na_2SO_4, and evaporated. The desired product was isolated as a yellowish liquid (3.25 g, 17.9 mmol, 82% yield) and was used for the next synthetic step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.30 (s, 6H), 3.85 (s, 2H), 5.36 (s, 1H), 7.28-7.40 (m, 4H, AA'BB'); ESI-MS: calcd for $C_{10}H_{16}NO_2$ ($[M+H]^+$) m/z 182.23, found 182.10.

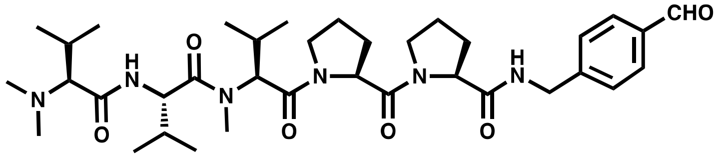
N,N-dimethylvalyl-valyl-*N*-methylvalyl-prolyl-proline (P5)



The pentapeptide P5 was assembled by step-wise solid phase peptide synthesis. Commercially available pre-loaded Fmoc-Pro on Tentagel resin (3.88 g, 0.66 mmol, RAPP polymere) was first swollen in DCM, then in DMF (2 x 3 min x 15 mL, in both cases). The Fmoc group was removed with 20 % piperidine in DMF (2 x 5 min x 10 mL). The resin was washed with DMF.

Prior coupling to the resin, amino acids were activated: 3 equiv. of each amino acid were pre-activated for 10 minutes with equimolar amounts of a coupling agent in the presence of 6 equiv. *N,N*-diisopropylethylamine (DIEA) at a concentration of 0.15 M in DMF. In the case of Fmoc-Pro-OH and Fmoc-N(Me)-Val-OH single couplings were performed using HBTU/HOBt; in the case of Fmoc-Val-OH and $(Me)_2$-Val-OH prolonged triple and double couplings respectively were required with HATU for the completion of the reaction. The pentapeptide was cleaved from the resin after 2h incubation with 60 mL of DCM/TFE/AcOH (3/1/1). The resin was filtered, the solvent evaporated and the desired product lyophilized from dioxane (310 mg, 85% yield; HPLC retention time: 9.15 min., Synergi column, 10-100% B in 15 minutes). ESI-MS: calcd for $C_{28}H_{5}0N_5O_6$ ($[M+H]^+$) *m/z* 552.7, found 552.1.

Cem-CHO



In a 25 mL round bottom flask containing P5 (50 mg, 0.091 mmol) dissolved in DMF (4 mL), HATU (34.6 mg, 0.091 mmol, 1 equiv.) and DIEA (63 µL, 0.36 mmol, 4 equiv.) were added. After 10 minutes pre-activation (4-(dimethoxymethyl)phenyl)methanamine (49.3 mg, 0.272 mmol, 3 equiv.) was added as a liquid. The reaction was complete after 1 hour as judged by HPLC. DMF was removed under high-vacuum, the crude re-suspended in 50 % CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (55 mg, 91 % yield, HPLC retention time: 12.87 min, Synergi column, 10-100% B in 25 min, 4 mL/min). ESI-MS: calcd for $C_{36}H_{57}N_6O_6$ ($[M+H]^+$) *m/z* 669.87, found 669.13. ^{1}H-NMR (500 MHz, DMSO) δ 0.69 (d, 3H, *j*= 6.3 Hz), 0.75-1.03 (m, 15H), 1.60-2.36 (m, 11H), 2.76 (s, 6H), 3.07 (s, 3H), 3.45-3.8 (m, 5H), 3.6-3.8 (m, 4H), 4.1-4.6 (m, 5H), 4.98 (d, 1H J= 10.4 Hz), 7.44-7.46 (m, 2H), 7.82-7.84 (m, 2H), 8.42-8.46 (m, 1H), 8.9-9.0 (m, 1H), 9.96 (s, 1H).

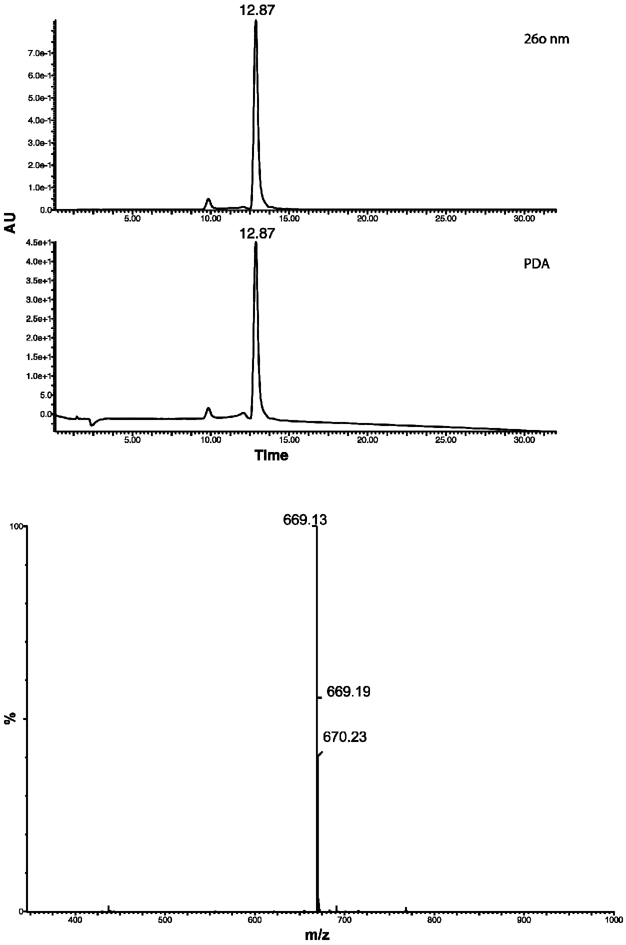


Figure SI2 HPLC trace and LC-MS spectrum of CemCHO.

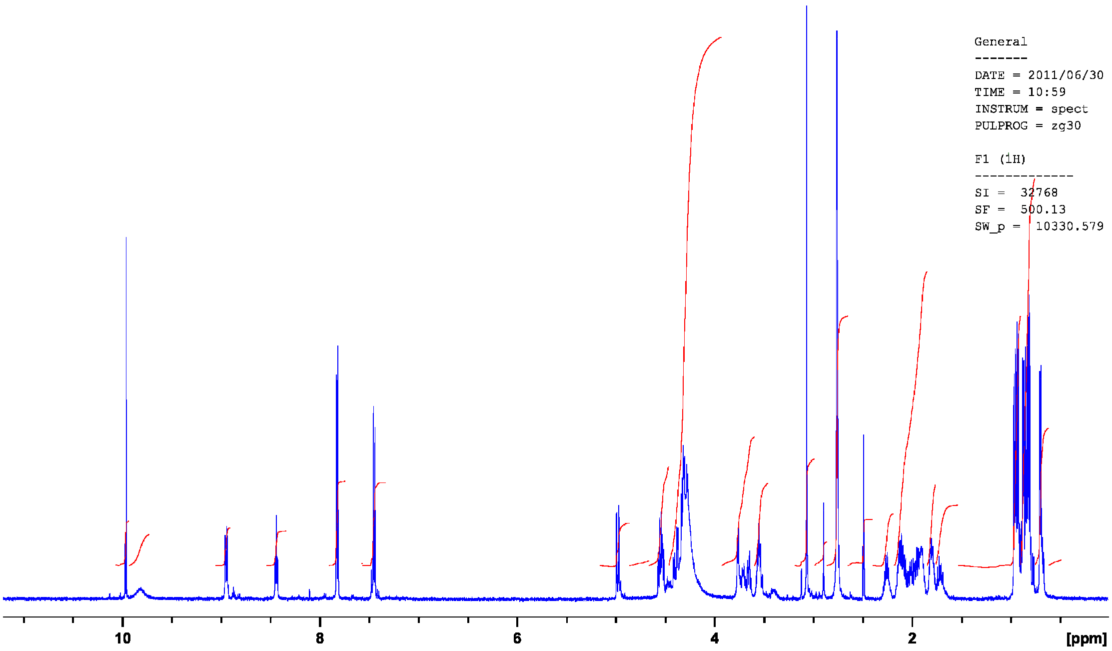


Figure SI3. ^{1}H-NMR spectrum of Cem-CHO.

2. Cloning, expression, purification and characterization of Cys-Db(F8) and Cys-Gly-Db(F8)

The diabody F8 (Db(F8)) sequence was modified in order to introduce an additional cysteine at the N-terminus, including glycine spacers units, upstream the diabody sequence.

The final constructs were double digested with NotI and HindIII, and ligated into a doubly digested pcDNA3.1.

2.1 Cloning strategy

Cys-dB(F8)

HindIII – *Leader Sequence* – **Cys** – V_H (F8) – *5aa (linker)* – V_L (F8) – **STOPSTOP – NotI**

DNA Sequence

CCC**aagctt***GTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTG*
*GCTACAGGTGTGCACTCG***TGC**GAGGTGCAGCTGTTGGAGTCTGGGGGAGG
CTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGG
GGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTAC
GCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAA
CACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTAT
ATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGG
GAACCCTGGTCACCGTCTCGAGT*GGCGGTAGCGGAGGG*GAAATTGTGTTG
ACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCT
CTCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCA
GCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCA
GGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGAC
TTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTAC
TGTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGGACCAAGGT
GGAAATCAAA**taaGCGGCCGC**AAAAGGAAAA

Protein sequence

*MGWSLILLFLVAVATGVHS***C**EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTM
SWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS*GGSGG*EIVLTQSPGTLSLSP
GERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS
GTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK

Secreted protein sequence (Cys-dB(F8))

CEVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAI
SGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLY
LFDYWGQGTLVTVSS*GGSGG*EIVLTQSPGTLSLSPGERATLSCRASQSVSMPF
LAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVY
YCQQMRGRPPTFGQGTKVEIK.
MW: 24890.0
pI: 8.81

Cys-Gly-dB(F8)

HindIII – *Leader Sequence* – **Cys** – **Gly** – V_H (F8) – *5aa (linker)* – V_L (F8) – **STOPSTOP – NotI**

DNA Sequence

CCCaagctt*GTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTG*
*GCTACAGGTGTGCACTCG***TGCGGT**GAGGTGCAGCTGTTGGAGTCTGGGGG
AGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTG
GATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGG
AAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATA
CTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGC
CGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGG
CCAGGGAACCCTGGTCACCGTCTCGAGT*GGCGGTAGCGGAGGG*GAAATTG
TGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGG

TACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATC
CAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGA
CAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTG
TATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGGAC
CAAGGTGGAAATCAAA**taaGCGGCCGC**AAAAGGAAAA

Protein sequence

MGWSLILLFLVAVATGVHS**C**GEVQLLESGGGLVQPGGSLRLSCAASGFTFSL
FTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQ
MNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGSGGEIVLTQSPGTL
SLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFS
GSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK

Secreted protein sequence (Cys-Gly-dB(F8))

CGEVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVS
AISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTH
LYLFDYWGQGTLVTVSSGGSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSM
PFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFA
VYYCQQMRGRPPTFGQGTKVEIK.
MW: 24947.0
pI: 8.81

2.2 Protein characterization

2.2.1 SDS-PAGE

Antibodies were analyzed on SDS-PAGE under non-reducing and reducing conditions. Proteins were mixed with reducing (20 mM DTT final concentration) or non-reducing 5 x SDS loading buffer. PageRuler^{TM} Plus prestained Protein (Fermentas, 00036845) served as a molecular marker. The samples were loaded on a precast NuPAGE® 10 – 12% Bis-Tris Gel (1.0 mm) (Invitrogen NP0315BOX) and run at 180 V and 110 mA for 50 minutes. NuPAGE® MES SDS Running Buffer 20x (Invitrogen NP0002) diluted in deionized H_2O was used as the corresponding buffer. Afterwards the gel was stained in Coomassie blue staining solution for 30 minutes followed by destaining.

2.2.2 Protein Mass Spectrometry

LC-MS was performed on a Micromass Quattro API instrument (ESI-MS) coupled to a Waters Alliance 2795 HPLC using a MassPREP desalting Cartridge 2.1 x 10 mm. Linear gradients of water with 0.1% formic acid (solvent A) and acetonitrile (solvent B), were used at a flow rate of 0.3 mL min^{-1}: 95% A (0.5 min isocratic) to 20% A after 1.5 min then isocratic till 3 min. Then in 1 min to 95% A and finally isocratic for 6 min. The electrospray source of LCT was operated with a capillary voltage of 3.0 kV and a cone voltage of 20 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h.

Proteins typically elute on a single peak between 3 and 4.5 minutes using this method. For conjugation analysis, the mass spectra corresponding to all protein in this peak were combined using MassLynx software (v. 4.0 from Waters). Mass spectra were calibrated with a minimum of 16 matched peaks from the multiply charged ion series of equine myoglobin (SIGMA, M0630), also measured at a cone voltage of 20 V. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.0 from Waters) according to manufacturer's instructions. The relative peak height that result from the reconstruction from total ion series is then used to calculate the relative amount of each protein and conjugation conversions. We assume that both modified and non-modified antibodies are ionized with similar efficiency.

2.2.3 Gel filtration analysis

The purified antibodies (normally 100 µL) as well as the antibody drug conjugates (ADCs) were analysed by FPLC on a Pharmacia ÄKTA-FPLC system (Amersham Pharmacia Biotech). A Superdex 200 HR 10/300 or a Superdex 75 10/300 GL column were used to perform gel filtration. The columns were run with PBS at a flow rate of 0.5 mL/min with UV detection (280 nm).

2.2.4 Biacore

The immunoreactivity of Db(F8) and the corresponding cemadotin conjugates was studied by surface plasmon resonance measurements on a Biacore 3000 system (GE Healthcare). CM5 microsensor chips were preactivated with EDC / NHS, and subsequently modified with the recombinant 11A12 fibronectin. The antibody fragments and derivatives were injected in different concentrations at a flow rate of 20 μL/min on a high-density coated 11A12-chip (3000 RU).

3. Site specific incorporation 1,2-aminothiol functionality in a protein sequence

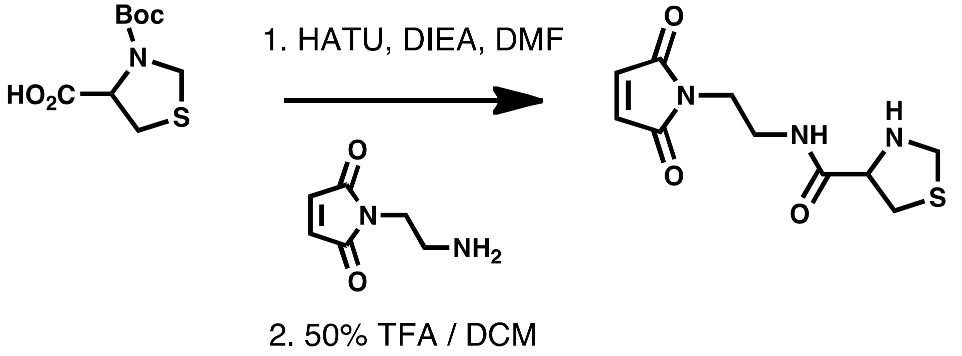
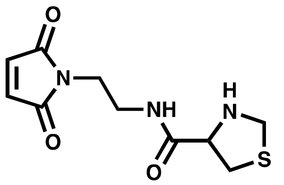


Figure SI4. Scheme for the synthesis of the maleimido/thiazolidine (M-thz) bifunctional linker.

3.1 Synthesis of N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)thiazolidine-4-carboxamide



In a 10 mL round bottom flask containing Boc-thiazolidine (100 mg, 0.429 mmol) dissolved in DMF (3 mL), HATU (155 mg, 0.407 mmol, 0.95 equiv.) and DIEA (225 μL, 1.29 mmol, 3 equiv.) were added. After 10 minutes pre-activation 1-(2-aminoethyl)-1*H*-pyrrole-2,5-dione (108 mg, 0.429 mmol, 1 equiv.) was added as a solid. The reaction was complete after 3 h at room temperature by HPLC chromatography. DMF was removed under vacuum, the crude re-suspended in 50% CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized. The white powder isolated was then dissolved in 50 % TFA in DCM (10 mL). After 40 minutes Boc deprotection was completed, the reaction evaporated, and the desired product lyophilized, and used without further purification (77 mg, 70 %, yield over two steps, HPLC retention time: 11.08 minutes, Synergi column, 20-80% B in 15 minutes, 4 mL/minute). ESI-MS: calcd for $C_{10}H_{14}N_3O_3S$ ($[M+H]^+$) *m/z* 256.3, found 255.7. ^{1}H-NMR (300 MHz, D_2O) δ 3.13 (dd, 1H, J=4.5 Hz, 9.3 Hz), 3.27-3.34 (m, 1H), 3.42 (dd, 1H, J=5.5 Hz, 9.3 Hz), 3.62-3.66 (m, 3H), 4.33 (d, 1H, J= 7.65 Hz), 4.40 (d, 1H, J= 7.65 Hz), 4.52 (dd, 1H, J= 0.99 Hz, 4.47 Hz), 6.82 (s, 2H).

3.2 Cem-CHO release from Db(F8)-Cys-M-thz-Cem ADC

The conjugate was tested for Cem-CHO release at 37 °C by tandem LC-MS/MS spectroscopy (**Figure SI5**). The half-life of the conjugate was 20 hours with the total amount of released drug measured to be 94% of the theoretical maximal amount of free Cem-CHO.

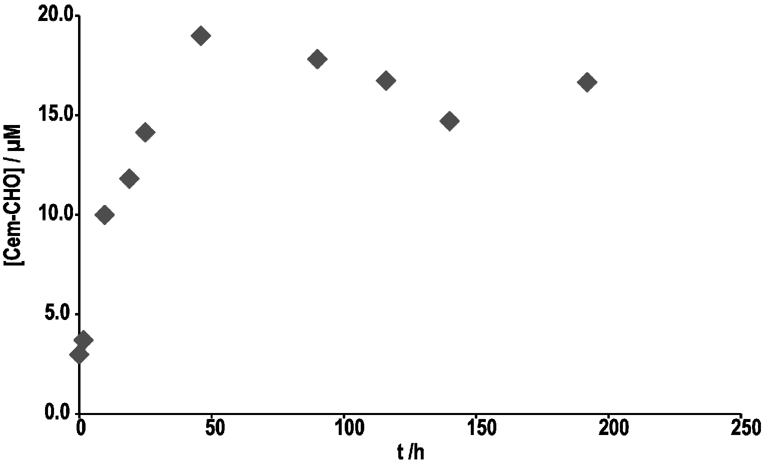


Figure SI5: Cem-CHO release at 37 °C in PBS, from Db(F8)Cys-M-thz-Cem. Starting ADC concentration of 20 µM.

4. Stability studies performed on Cem-thz-Gly-Db(F8).

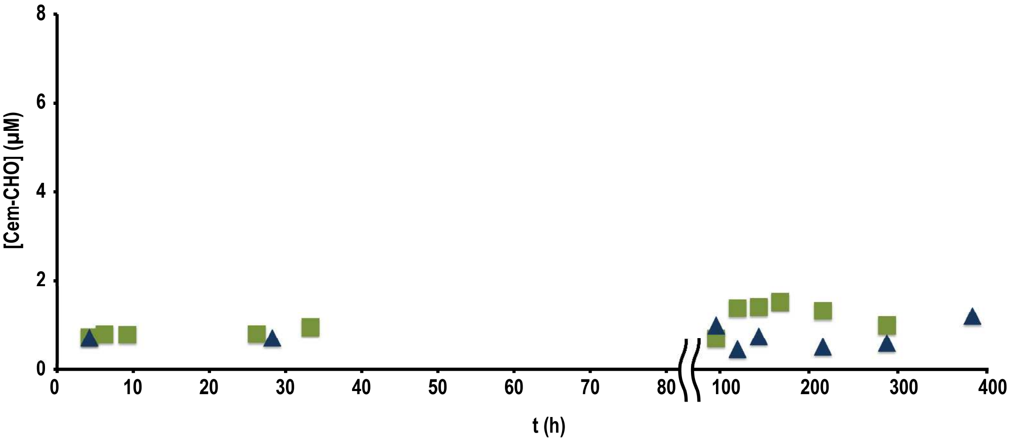


Figure SI6: Stability studies performed on Cem-thz-Gly-Db(F8). Cem-CHO released was measured as described in the methods section of the main article. Samples were stored at 4 °C (green) and – 80 °C (blue).

5. Non-cleavable cemadotin-antibody conjugates.

Synthesis of cemadotin thioester (Cem-COSR)

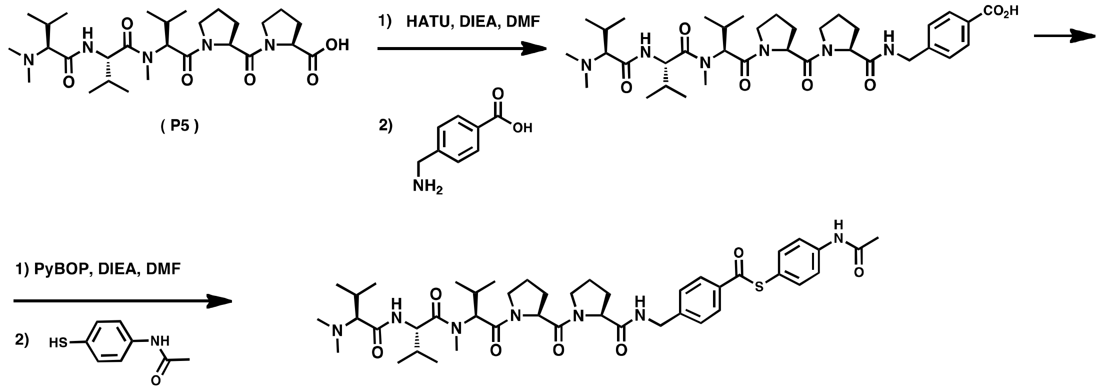
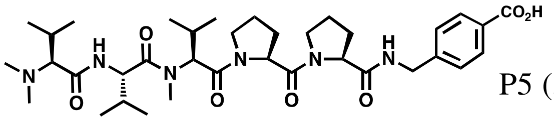


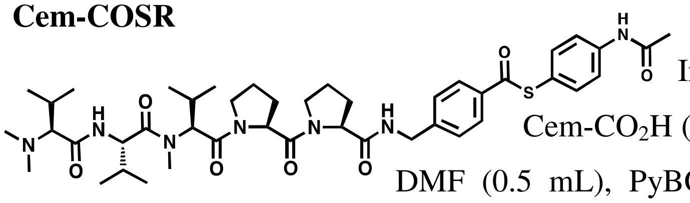
Figure SI7. Synthesis of cemadotin thioester analog, Cem-COSR

Cem-CO$_2$H



In a 25 mL round bottom flask containing P5 (30 mg, 0.054 mmol) dissolved in DMF (1.5 mL), HATU (18.6 mg, 0.049 mmol, 0.9 equiv.) and DIEA (19 μL, 0.11 mmol, 2 equiv.) were added. After 10 minutes pre-activation, 4-(aminomethyl)-benzoic acid (9.04 mg, 0.06 mmol, 1.1 equiv.) was added. The reaction was judged completed after 2 h by HPLC chromatography. DMF was removed under vacuum, the crude re-suspended in 50% CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (26 mg, 77 %, HPLC retention time: 8.2 min., Synergi column, 20-80% B in 15 min., 4 mL/min.). ESI-MS: calcd for $C_{36}H_{57}N_6O_7$ ($[M+H]^+$) *m/z* 685.9, found 685.4.

Cem-COSR



In a 1.5 mL eppendorf containing Cem-CO$_2$H (25 mg, 0.037 mmol) dissolved in DMF (0.5 mL), PyBOP (28.5 mg, 0.055 mmol, 1.5 equiv.), DIEA (9.6 μL, 0.055 mmol, 1.5 equiv.) and *p*-acetamido thiophenol (9.2 mg, 0.055 mmol, 1.5 equiv.) were added. The reaction was judged completed after 2 h by HPLC. DMF was removed under vacuum, the crude re-suspended in 50% CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (25 mg, 82 % yield, HPLC retention time: 12.55 min., Synergi column, 20-80% B in 15 min., 4 mL/min.). ESI-MS: calcd for $C_{44}H_{64}N_7O_7S$ ($[M+H]^+$) *m/z* 835.1, found 834.6.

Native chemical ligation (NCL)

In a typical experiment the pre-treated antibody (0.2-1 mg/mL, 8-40 μM, see methods section in main article) was reacted with cemadotin thioester (Cem-COSR, 50 equiv.) in the presence of sodium-mercaptoethanesulfonate (MESNA, 10 mM) and TCEP (15 mM) in PBS buffer (pH 7) in the absence of denaturants. Both MESNA and TCEP were premixed as a 10x stock and pH adjusted to 7 prior to use. Typically after 48 hours full conversion was observed as judged by ESI-MS (**Figure SI8**)

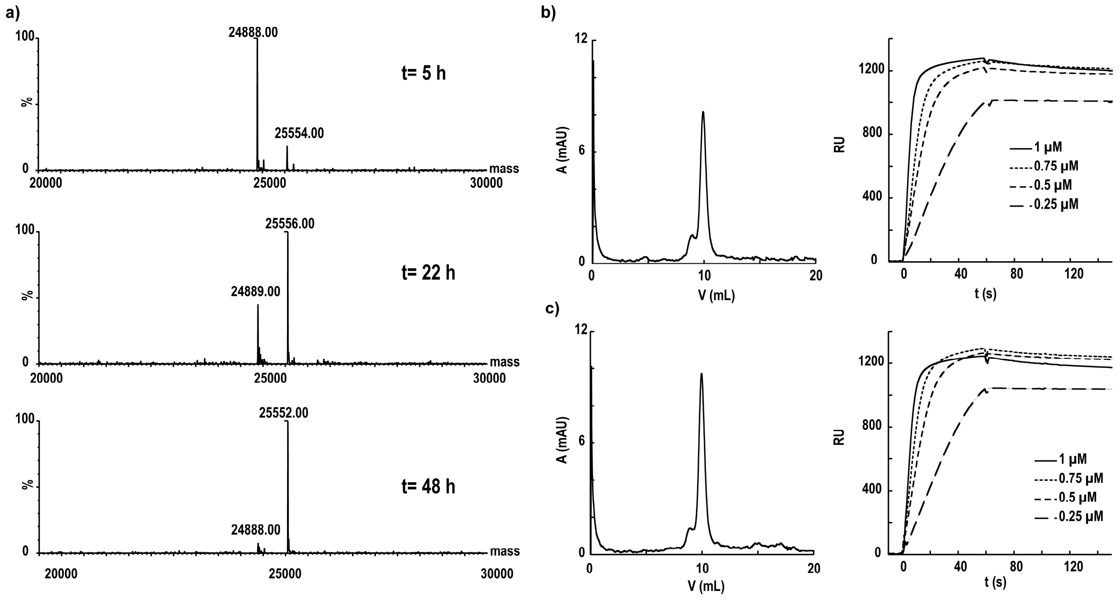


Figure SI8: NCL reaction analysis. a) Time-course analysis of the NCL reaction between Cem-COSR and Cys-Db(F8). ESI-MS: calcd for Cys-Db(F8) = 24890.0 Da; calcd for Cem-CONH-Cys-Db(F8) = 25556.9 Da; For Cys-Gly-Db(F8) 5 days were required to observe 70 % conversion; in this case 10 % of doubly labelled antibody was observed. b-c) FPLC and Biacore analysis of Cem-CONH-Cys-Db(F8) and Cem-CONH-Cys-Gly-Db(F8) respectively.

6. References

(1) Flamigni, L.; Ventura, B.; Tasior, M.; Becherer, T.; Langhals, H.; Gryko, D. T. *Chem. Eur. J.* **2008**, *14*, 169.