Parallel Synthesis and Screening of Peptide Conjugates

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SUPPLEMENTARY TABLE S1

Table S1. Protocol for the synthesis of the peptides in the 96-vessel block format.

Step	Reagents and Operation	Time (minutes)	Repetition
1	Wash with 200 µL of DMF	1	6 times
2	80 µL of 0.5 M Fmoc-protected amino acid in DMF 80 µL of 0.5 M HCTU in DMF 40 µL of 2 M NMM in DMF		
3	Coupling	20	
4	Empty	1	
5	80 µL of 0.5 M Fmoc-protected amino acid in DMF 80 µL of 0.5 M HCTU in DMF 40 µL of 2 M NMM in DMF		
6	Coupling	20	
7	Empty		
8	Wash with 200 µL of DMF	1	5 times
9	200 µL 20 v-% piperidine in DMF	5	2 times
10	Empty	1	

SUPPLEMENTARY TABLE S2

Table S2. Peptide sequences and their purified recovered yields.

Well ID	Amino acid sequence	Purified recovered yield (mg)
	TETHER WALK – Lys NOT ACETYLATED	
A01	**K(N_3)**KYQPLDELD**K**TLYDQFMLQQG	1
A02	Q**K(N_3)**YQPLDELD**K**TLYDQFMLQQG	1.6
A03	Q**K****K(N_3)**QPLDELD**K**TLYDQFMLQQG	1.4
A04	Q**K**Y**K(N_3)**PLDELD**K**TLYDQFMLQQG	1.4
A05	Q**K**YQ**K(N_3)**LDELD**K**TLYDQFMLQQG	1
A06	Q**K**YQP**K(N_3)**DELD**K**TLYDQFMLQQG	1.2
A07	Q**K**YQPL**K(N_3)**ELD**K**TLYDQFMLQQG	1.2
A08	Q**K**YQPLD**K(N_3)**LD**K**TLYDQFMLQQG	0.8
A09	Q**K**YQPLDE**K(N_3)**D**K**TLYDQFMLQQG	0.7
A10	Q**K**YQPLDEL**K(N_3)****K**TLYDQFMLQQG	1.8
A11	Q**K**YQPLDELD**K(N_3)**TLYDQFMLQQG	0.8
A12	Q**K**YQPLDELD**K****K(N_3)**LYDQFMLQQG	1.2
B01	Q**K**YQPLDELD**K**T**K(N_3)**YDQFMLQQG	1.4
B02	Q**K**YQPLDELD**K**TL**K(N_3)**DQFMLQQG	1
B03	Q**K**YQPLDELD**K**TLY**K(N_3)**QFMLQQG	1.6
B04	Q**K**YQPLDELD**K**TLYD**K(N_3)**FMLQQG	0.7
B05	Q**K**YQPLDELD**K**TLYDQ**K(N_3)**MLQQG	1.1
B06	Q**K**YQPLDELD**K**TLYDQF**K(N_3)**LQQG	1.5
B07	Q**K**YQPLDELD**K**TLYDQFM**K(N_3)**QQG	1.4
B08	Q**K**YQPLDELD**K**TLYDQFML**K(N_3)**QG	0.8
B09	Q**K**YQPLDELD**K**TLYDQFMLQ**K(N_3)**G	1

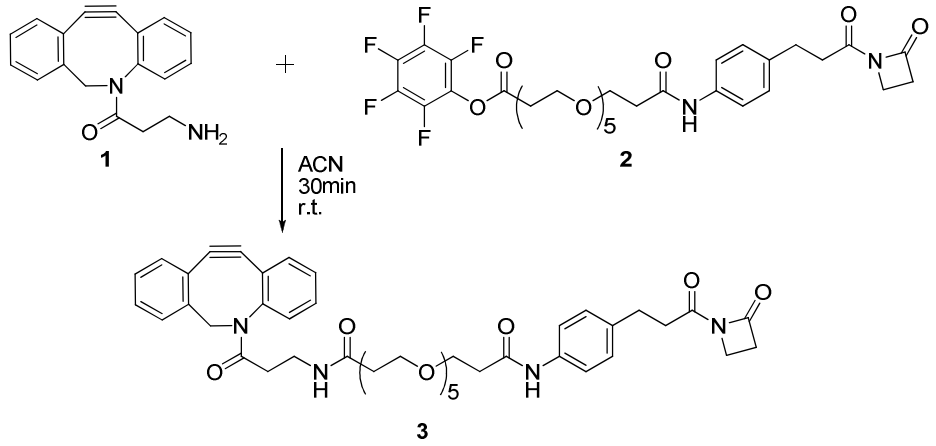
B10	Q**K**YQPLDELD**K**TLYDQFMLQQ**K(N_3)**	0.7
	TETHER WALK – Lys ACETYLATED	
B11	**K(N_3)**K(Ac)YQPLDELDK(Ac)TLYDQFMLQQG	0.7
B12	Q**K(N_3)**YQPLDELDK(Ac)TLYDQFMLQQG	1.5
C01	QK(Ac)**K(N_3)**QPLDELDK(Ac)TLYDQFMLQQG	0.6
C02	QK(Ac)Y**K(N_3)**PLDELDK(Ac)TLYDQFMLQQG	0.9
C03	QK(Ac)YQ**K(N_3)**LDELDK(Ac)TLYDQFMLQQG	0.8
C04	QK(Ac)YQP**K(N_3)**DELDK(Ac)TLYDQFMLQQG	0.9
C05	QK(Ac)YQPL**K(N_3)**ELDK(Ac)TLYDQFMLQQG	1
C06	QK(Ac)YQPLD**K(N_3)**LDK(Ac)TLYDQFMLQQG	1.1
C07	QK(Ac)YQPLDE**K(N_3)**DK(Ac)TLYDQFMLQQG	1.2
C08	QK(Ac)YQPLDEL**K(N_3)**K(Ac)TLYDQFMLQQG	2
C09	QK(Ac)YQPLDELD**K(N_3)**TLYDQFMLQQG	0.7
C10	QK(Ac)YQPLDELDK(Ac)**K(N_3)**LYDQFMLQQG	0.9
C11	QK(Ac)YQPLDELDK(Ac)T**K(N_3)**YDQFMLQQG	0.7
C12	QK(Ac)YQPLDELDK(Ac)TL**K(N_3)**DQFMLQQG	0.4
D01	QK(Ac)YQPLDELDK(Ac)TLY**K(N_3)**QFMLQQG	0.7
D02	QK(Ac)YQPLDELDK(Ac)TLYD**K(N_3)**FMLQQG	1.5
D03	QK(Ac)YQPLDELDK(Ac)TLYDQ**K(N_3)**MLQQG	1.1
D04	QK(Ac)YQPLDELDK(Ac)TLYDQF**K(N_3)**LQQG	1.3
D05	QK(Ac)YQPLDELDK(Ac)TLYDQFM**K(N_3)**QQG	1.1
D06	QK(Ac)YQPLDELDK(Ac)TLYDQFML**K(N_3)**QG	0.8
D07	QK(Ac)YQPLDELDK(Ac)TLYDQFMLQ**K(N_3)**G	0.5
D08	QK(Ac)YQPLDELDK(Ac)TLYDQFMLQQ**K(N_3)**	0.7
	ALANINE SCAN – Lys ACETYLATED	
D09	AK(Ac)YQPLDELD**K(N_3)**TLYDQFMLQQG	0.7
D10	QAYQPLDELD**K(N_3)**TLYDQFMLQQG	1.1

D11	QK(Ac)**A**QPLDELD**K(N$_3$)**TLYDQFMLQQG	0.7
D12	QK(Ac)Y**A**PLDELD**K(N$_3$)**TLYDQFMLQQG	0.5
E01	QK(Ac)YQ**A**LDELD**K(N$_3$)**TLYDQFMLQQG	0.9
E02	QK(Ac)YQP**A**DELD**K(N$_3$)**TLYDQFMLQQG	0.7
E03	QK(Ac)YQPL**A**ELD**K(N$_3$)**TLYDQFMLQQG	1.4
E04	QK(Ac)YQPLD**A**LD**K(N$_3$)**TLYDQFMLQQG	0.5
E05	QK(Ac)YQPLDE**A**D**K(N$_3$)**TLYDQFMLQQG	1.2
E06	QK(Ac)YQPLDEL**A**K**(N$_3$)**TLYDQFMLQQG	0.5
E07	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQFMLQQG	0.7
E08	QK(Ac)YQPLDELD**K(N$_3$)A**LYDQFMLQQG	0.8
E09	QK(Ac)YQPLDELD**K(N$_3$)**T**A**YDQFMLQQG	0.8
E10	QK(Ac)YQPLDELD**K(N$_3$)**TL**A**DQFMLQQG	0.3
E11	QK(Ac)YQPLDELD**K(N$_3$)**TLY**A**QFMLQQG	0.8
E12	QK(Ac)YQPLDELD**K(N$_3$)**TLYD**A**FMLQQG	0.4
F01	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQ**A**MLQQG	1
F02	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQF**A**LQQG	0.6
F03	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQFM**A**QQG	0.9
F04	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQFML**A**QG	0.3
F05	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQFMLQ**A**G	0.4
F06	QK(Ac)YQPLDELD**K(N$_3$)**TLYDQFMLQQ**A**	0.3
MUTANTS AT POSITION 09 – Lys ACETYLATED		
F07	QK(Ac)YQPLDE**L**D**K(N$_3$)**TLYDQFMLQQG	0.7
F08	QK(Ac)YQPLDE**G**D**K(N$_3$)**TLYDQFMLQQG	0.5
F09	QK(Ac)YQPLDE**H**D**K(N$_3$)**TLYDQFMLQQG	0.3
F10	QK(Ac)YQPLDE**I**D**K(N$_3$)**TLYDQFMLQQG	0.5
F11	QK(Ac)YQPLDE**K(Ac)**D**K(N$_3$)**TLYDQFMLQQG	0.7
F12	QK(Ac)YQPLDE**K(Ac)**D**K(N$_3$)**TLYDQFMLQQG	0.3

G01	QK(Ac)YQPLDE**N**D**K(N_3)**TLYDQFMLQQG	0.9
G02	QK(Ac)YQPLDE**P**D**K(N_3)**TLYDQFMLQQG	1.2
G03	QK(Ac)YQPLDE**Q**D**K(N_3)**TLYDQFMLQQG	0.3
G04	QK(Ac)YQPLDE**R**D**K(N_3)**TLYDQFMLQQG	0.5
G05	QK(Ac)YQPLDE**S**D**K(N_3)**TLYDQFMLQQG	0.3
G06	QK(Ac)YQPLDE**T**D**K(N_3)**TLYDQFMLQQG	0.8
G07	QK(Ac)YQPLDE**V**D**K(N_3)**TLYDQFMLQQG	0.3
G08	QK(Ac)YQPLDE**B**D**K(N_3)**TLYDQFMLQQG	0.4
G09	QK(Ac)YQPLDE**O**D**K(N_3)**TLYDQFMLQQG	0.2
	PEPTIDE-AZIDE CONTROLS	
H01	QK(Ac)YQPLDE**K(Ac)**D**K(N_3)**TLYDQFMLQQG	
H02	QK(Ac)YQPLDE**K(Ac)**D**K(PEG$_4$$N_3$)**TLYDQFMLQQG	

Note: all peptides are acetylated at the N-terminus and have a C-terminal amide. B = Aib; O = Orn. **H01**, **F11**, and **F12** have the same peptide sequence. **C09**, **E07**, and **F07** have the same peptide sequence. **H01** and **H02** are synthesized separately from the plate by standard solid phase peptide synthesis protocols as they were designed to serve as references in the screen.

SUPPLEMENTARY SCHEME S1



Scheme S1. Synthesis of the DBCO-PEG$_5$-β-lactam linker (**3**).

SUPPLEMENTARY FIGURE S1

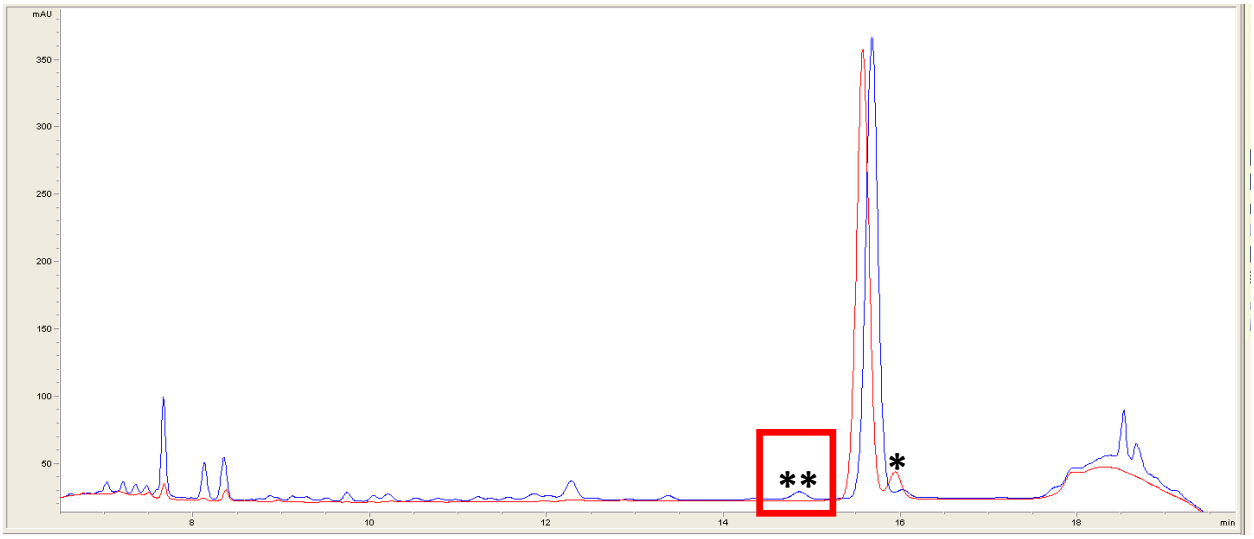


Figure S1. RP HPLC trace of the crude reaction mixture for the synthesis of DBCO-PEG_5-β-lactam linker (blue) overlaid with the RP HPLC trace of the purified DBCO-PEG_5-β-lactam linker (**3**) (red). Monitored at 214 nm (reference 360 nm). * Is a by-product of DBCO-PEG_5-β-lactam hydrolyzed at the β-lactam group, which does not interfere with the reaction to CVX-2000. ** Is the undesired by-product COOH-PEG_5-β-lactam (hydrolyzed at the DBCO amide), which generates "blocked" pockets on the CVX-2000 antibody scaffold.

SUPPLEMENTARY FIGURE S2

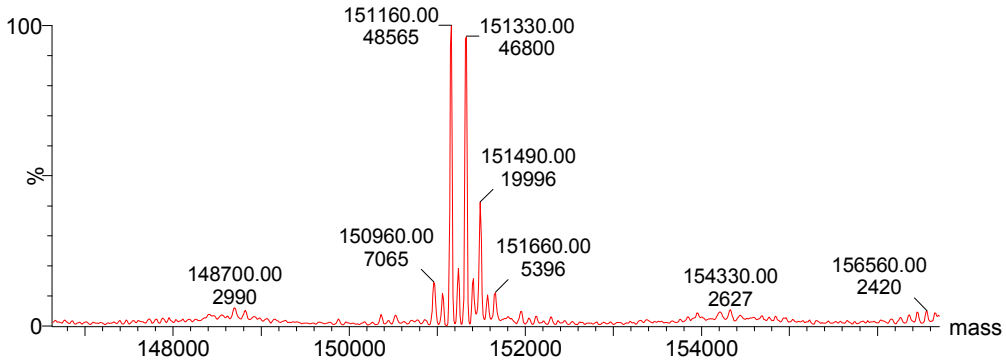


Figure S2. SEC-MS spectrum of CVX-2000-PEG_5-DBCO. Calculated average of 1.8 DBCO groups per CVX-2000 antibody scaffold.

SUPPLEMENTARY FIGURE S3

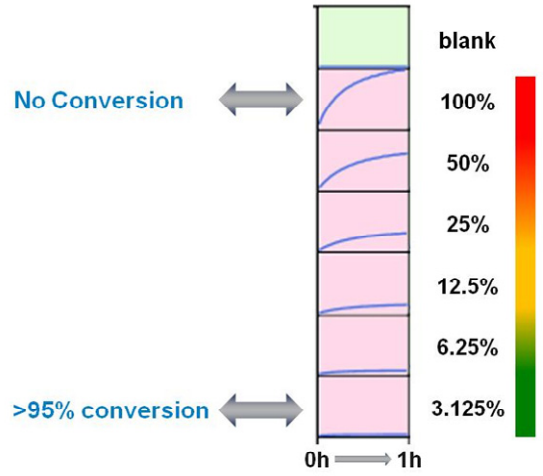


Figure S3. Standard curve for the Coumarin assay based on a serial dilution of CVX-2000-PEG_5-DBCO.

SUPPLEMENTARY FIGURE S4

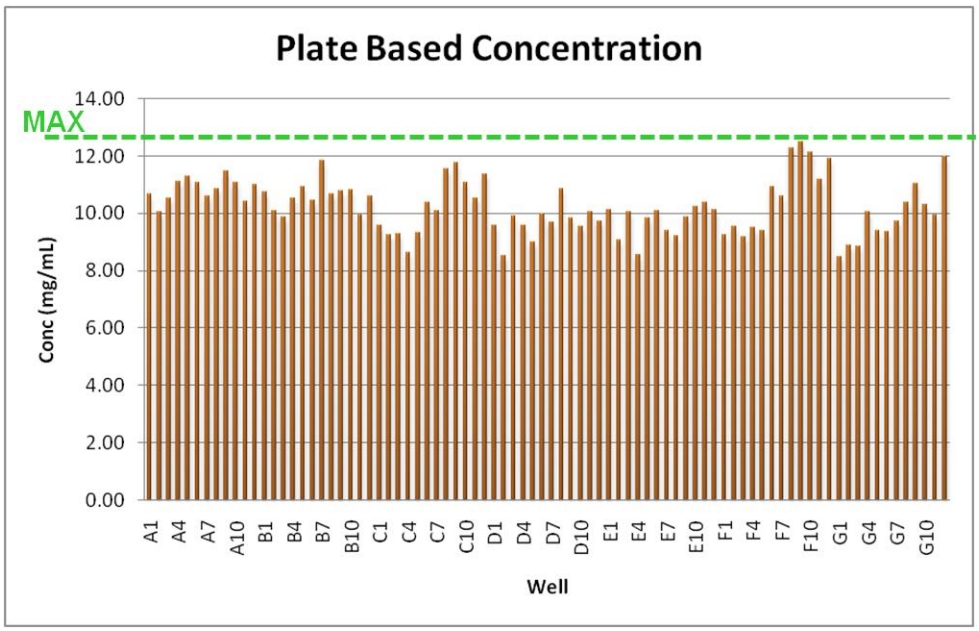


Figure S4. Concentration measurements for the peptide-antibody conjugates. **H01-H03** were transferred to wells **G10-G12** for this assay.

SUPPLEMENTARY FIGURE S5

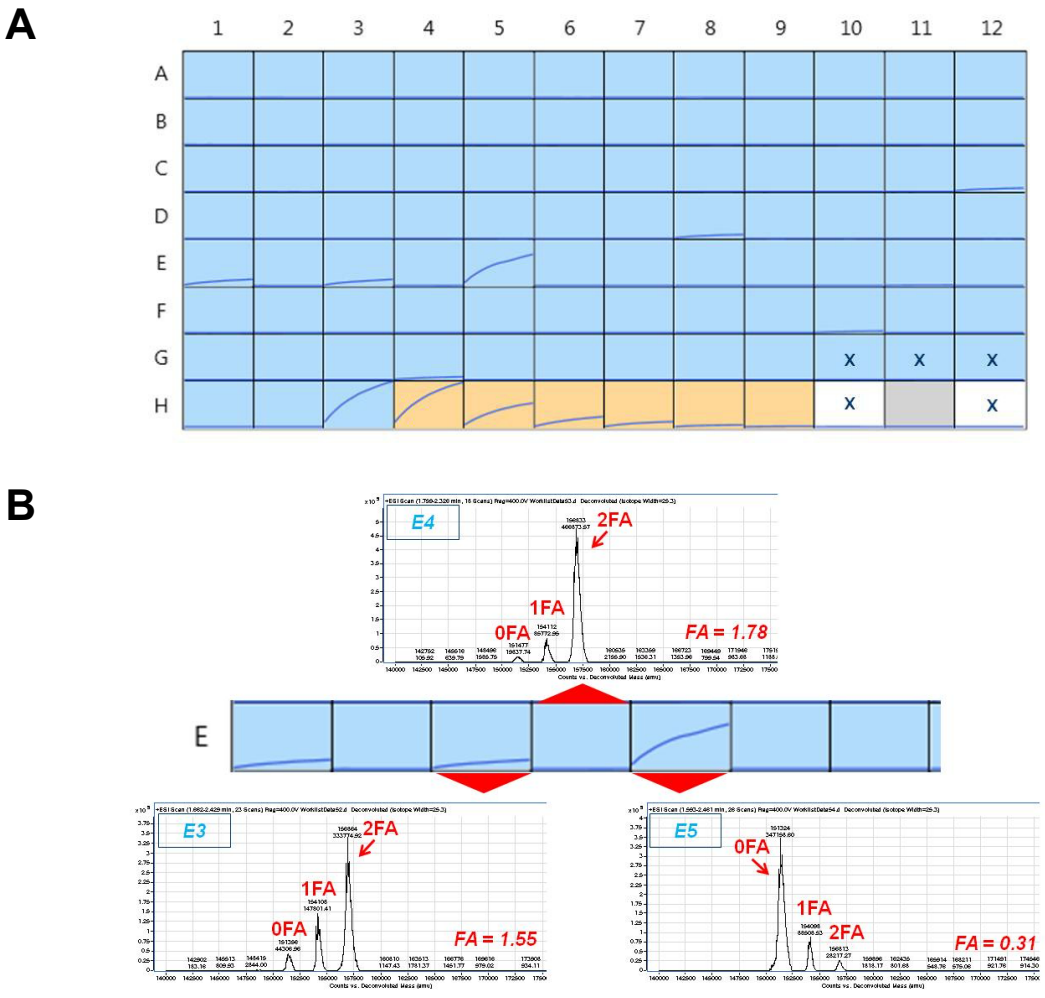


Figure S5. A Read-out of the Coumarin-assay performed in parallel for the 84 conjugation reactions (λ_{ex} = 404 nm, λ_{em} = 477 nm; every 5 minutes for 1 hour). Wells marked X = empty, orange = standard curve, grey = buffer.; **B** SEC-MS spectra of **E03**, **E04**, and **E05** after purification aligned with the results of the Coumarin assay that was performed prior to purification. FA = Functional Additions, the average number of peptides conjugated to the antibody scaffold.

SEC-MS analysis confirmed the initial read-out of the Coumarin assay. Representative examples are given in Supplementary Figure S5B. Maximum average number of peptides that can be conjugated to this batch of CVX-2000-PEG_5-DBCO is 1.8 (see also Supplementary Figure S2).

SUPPLEMENTARY FIGURE S6

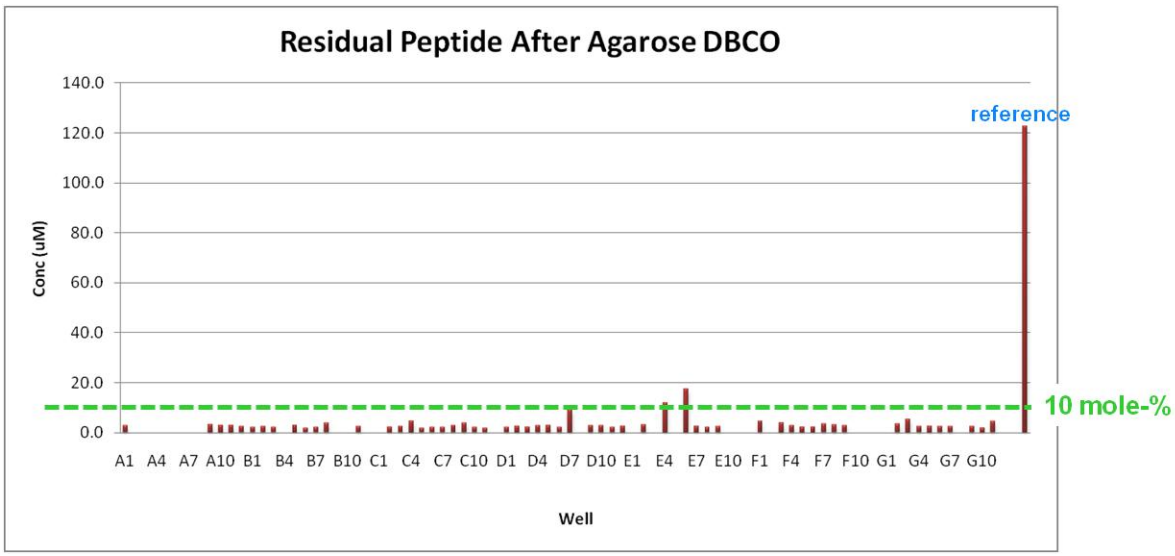


Figure S6. Residual peptide analysis of the peptide-antibody conjugates after purification with immobilized DBCO scavenger. **H01-H03** were transferred to **G10-G12** for this assay. Reference is crude **H02** peptide-antibody conjugation mixture in which excess peptide is present.

SUPPLEMENTARY FIGURE S7

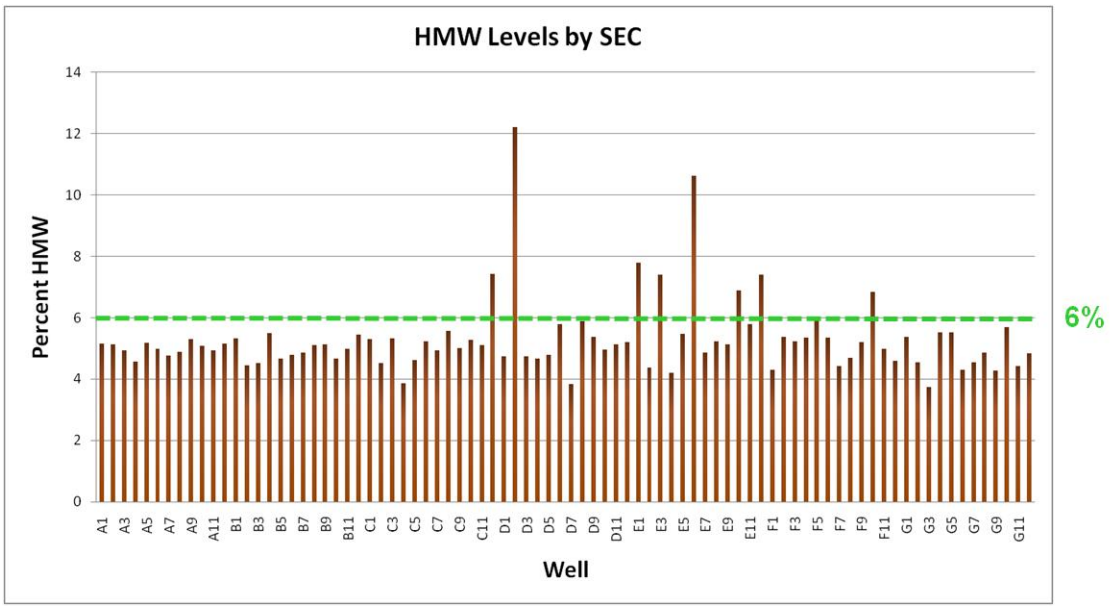
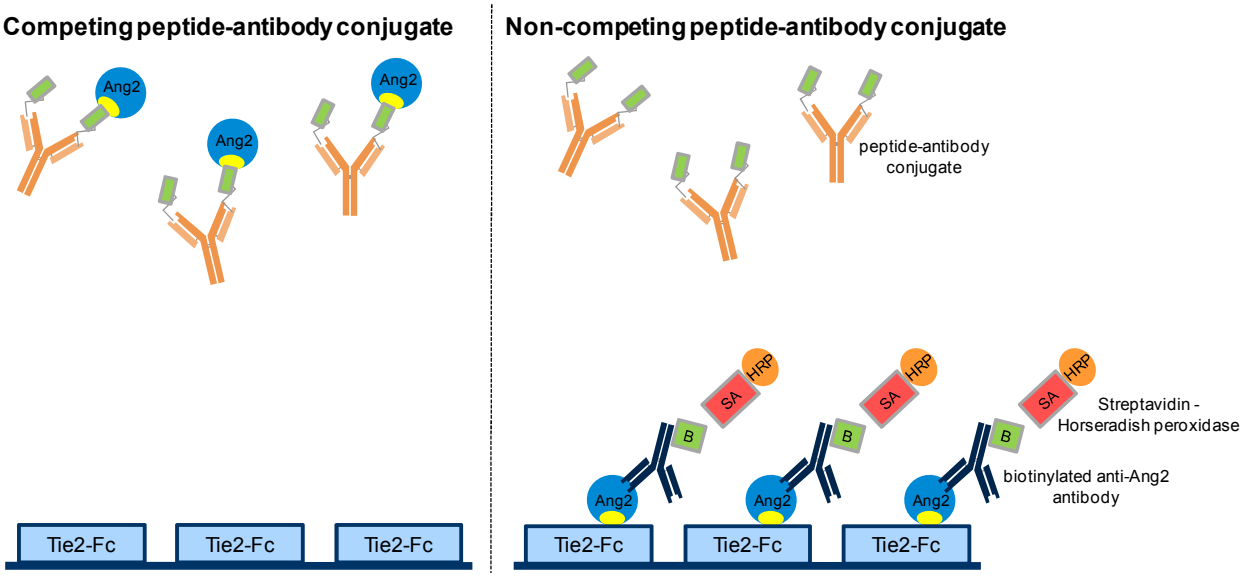


Figure S7. Aggregate analysis (HMW = high molecular weight) of the peptide-antibody conjugates. **H01**-**H03** were transferred to wells **G10**-**G12** for this assay.

SUPPLEMENTARY SCHEME S2



Scheme S2. Schematic representation of the Tie2/Ang2 competition assay. In the case of a strongly competing peptide-antibody conjugate, Ang2 will primarily bind to the peptide-antibody conjugate in solution (*left*). In the case of a non-competing peptide-antibody conjugate, Ang2 will primarily bind to the Tie2-Fc immobilized on the surface (*right*). After incubation and prior to detection, all components in solution are removed in a washing step. Ang2 bound to immobilized Tie2-Fc is detected using a biotinylated anti-Ang2 antibody followed by Streptavidin-Horseradish peroxidase.

SUPPLEMENTARY TABLE S3

Table S3. Ranking of the copper-free clicked peptide-CVX-2000 antibody conjugates: relative competition of the copper-free clicked peptide-CVX-2000 antibody conjugates to the **H02** reference (set at 1.0). Green = stronger competitors than **H02**; Light green = similar competitors as **H02**; Orange = less effective competitors than **H02**; Red = poorly or not competing.

Well ID	Relative competition (normalized to H02)
D01	> 6
E08	> 6
A02	> 6
B04	> 6
C10	> 6
B10	> 6
A12	> 6
B07	> 6
D05	6.0
D08	6.0
B09	6.0
B08	5.1
E07	4.7
B12	4.4
C09	4.3
B03	4.0
F03	3.5
F07	3.4
D07	2.8
A08	2.6
F06	2.4
F04	2.3
D10	2.2
D04	1.9
C06	1.7
D06	1.5
E12	1.3
D02	1.2
H02	1.0

E04	1.0
B06	0.9
F05	0.9
A09	0.7
A11	0.7
A01	0.6
G03	0.6
C07	0.5
D09	0.4
B11	0.4
G01	0.3
A04	0.3
H01	0.3
G08	0.2
E11	0.2
E01	0.2
G07	0.2
F11	0.2
C02	0.2
F12	0.2
G06	0.2
D12	0.2
F10	0.2
F01	0.1
F02	0.1
F09	0.1
C04	0.05
G02	0.05
E02	0.05
F08	0.05
G04	0.05
C05	0.05
C01	0.05
G05	0.05
D03	0.05
E05	0.05
E06	0.05
E09	0.05
E03	0.05
D11	0.05
B02	0.05
C11	0.05
E10	0.05

A03	0.05
B01	0.05
A05	0.05
A07	0.05
C03	0.05
G09	0.05
A10	0.05
A06	0.05
C08	0.05
H03	0.05
B05	0.05
C12	0.05
H12	0.05

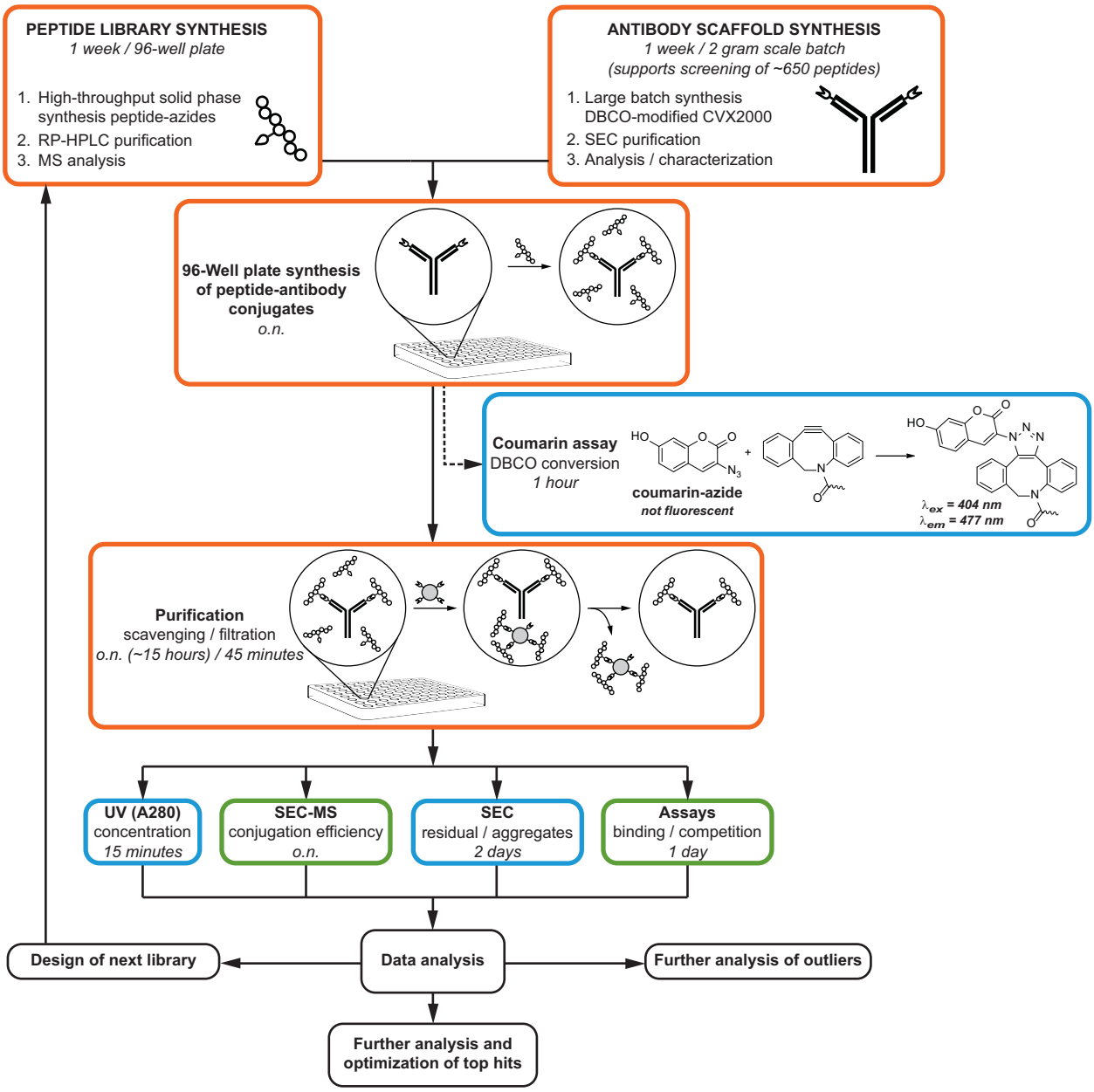
SUPPLEMENTARY TABLE S4

The IC_{50} values that were originally reported[1] were normalized to CVX-060 (Supplementary Table S4) to allow for a direct comparison of the outcome of the original optimization study using the traditional β-lactam fusion chemistry and the new parallel approach using copper free click chemistry. For this, the IC_{50} of CVX-060 is divided by the IC_{50} of each of the other peptide conjugates (the lower the IC_{50}, the stronger the peptide-antibody conjugate competes with Tie2 for binding to Ang2).

Table S4. Normalization of the original data set published previously.[1]

Well ID[1]	IC_{50} (nM)[1]	Relative competition (normalized to CVX-060)
12	0.1	5
15	0.2	2.5
19	0.1	5
22	0.2	2.5
2	0.3	1.7
CVX-32	0.3	1.7
21	0.3	1.7
18	0.6	0.8
8	0.3	1.7
20	0.1	5
16	0.3	1.7
9	2	0.3
1	1.8	0.3
CVX-060	0.5	1.0
4	>1000	0
17	19.5	0.03
14	32.8	0.02
5	44.2	0.01
3	0.2	2.5
6	>1000	0
13	>1000	0
10	>1000	0
7	>1000	0

SUPPLEMENTARY SCHEME S3



Scheme S3. Flowchart of the parallel approach to peptide conjugates as applied in the manuscript. It indicates the steps that can be performed in parallel and gives the timelines associated with it (**Orange**: improvement in robustness and throughput; **Blue**: newly developed analytical assays; **Green**: previously reported analytical assays[1] adapted for higher throughput).

1) Huang, H., Lai, J. –Y., Do, J., Li, L., Del Rosario, J., Doppalapudi, V. R., Pirie-Shepherd, S., Levin, N., Bradshaw, C., Woodnutt, G., Lappe, R., and Bhat, A. (2011) Specifically targeting Angiopoietin-2 inhibits angiogenesis, Tie2-expressing monocyte infiltration, and tumor growth. *Clin. Cancer Res. 17*, 1001–1011.