Chemically Programmed Bispecific Antibodies that Recruit and Activate T Cells*

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Running title: *Chemically programmed bispecific antibodies*

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Synthesis of chemical compounds - The synthesis of *LLP2A-biotin-maleimide* was described previously (1). The synthesis of *folate-biotin-maleimide* will be described elsewhere. Synthesis of *(LLP2A)2-biotin-maleimide*: NovaSyn TGR resin (0.2 mmol/g loading) was purchased from Novabiochem. All protected amino acids and peptide coupling reagents, with the exception of diisopropylcarbodiimide (DIC), were obtained from the following vendors: Novabiochem, Advanced ChemTech, and Chem-Impex International. All other reagents were obtained from Sigma-Aldrich. High-resolution mass spectra (HRMS) were measured by the University of California Riverside Mass Spectrometry Facility. Solid-phase syntheses were carried out in 10 mL reaction columns (Pierce) using a LabQuake shaker (Barnstead Thermolyne). Unless stated otherwise, the resin was washed with N-methyl-2-pyrrolidone (NMP) (6 x 7 mL) following each cycle of coupling, capping (with acetylimidazole), and deprotection. Coupling was performed at room temperature in NMP and monitored via the Kaiser test (1). A solution of 1-acetylimidazole (10 % (w/v) in dimethylformamide (DMF), 1-1.5 h) was used to acetylate or "cap" any unreacted amino groups on the resin at the end of each coupling step. Deprotection of Fmoc was accomplished with 20 % piperidine in DMF (2 x 15 min). Reaction products requiring HPLC purification were purified using a Waters PrepLC 4000 preparative HPLC system having photodiode array detection and using a Phenomenex C_{18} column (250 mm x 21 mm; 5-µm particle size, 110 Å pore size) at a flow rate of 10 mL/min. HPLC solvent system consisted of: Solvent A = 0.1 % (w/v) trifluoroacetic acid (TFA) in H_2O, Solvent B = 0.1 % (w/v) TFA in acetonitrile. To a pre-swollen (2 h) suspension of NovaSyn TGR resin (0.500 g, 0.100 mmol) in NMP was added Fmoc-Lys(Mtt)-OH (0.312 g, 0.500 mmol), *N*-hydroxybenzotriazole (HOBt; 0.068 g, 0.500 mmol), *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluroniumhexafluorophosphate (HBTU; 0.190 g, 0.500 mmol), and *N*,*N*-diisopropylethylamine (DIEA; 174 µL, 1.00 mmol). The mixture was agitated for 2 h at room temperature. In order to push the reaction to completion, the coupling was repeated overnight at room temperature with DIC (77.4 µL, 0.500 mmol), Fmoc-Lys(Mtt)-OH (0.500 mmol), and HOBt (0.500 mmol). A solution of Fmoc-Lys(biotin)-OH (0.297 g, 0.500 mmol), HOBt (0.500 mmol), HBTU (0.500 mmol), and DIEA (1.00 mmol) was added and the mixture was then agitated 4 h. A solution of Fmoc-Lys(Fmoc)-OH (0.591 g, 1.00 mmol), HOBt (0.135 g, 1.00 mmol), and DIC (155 µL, 0.126 g, 1.00 mmol) was added and the resin mixture was shaken overnight. A solution of Fmoc-Arg(Pbf)-OH (0.649 g, 1.00 mmol), HBTU (0.379 g, 1.00 mmol), HOBt (0.135 g, 1.00 mmol), and DIEA (349 µL, 2.00 mmol) was added and then the resin was shaken 3.5 h. A solution of Fmoc-PEG-SU (2) (0.471 g, 1.00 mmol), HOBt (0.135 g, 1.00 mmol), and DIC (155 µL, 1.00 mmol) was added to the resin and the mixture was then shaken overnight. The length of the PEG-SU linker was extended by allowing the resin to react 2 h with additional Fmoc-PEG-SU (0.471 g, 1.00 mmol), HOBt (0.135 g, 1.00 mmol), HBTU (0.379 g, 1.00 mmol), and DIEA (349 µL, 2.0 mmol). After capping and Fmoc-deprotection, LLP2A was synthesized at both *N*-termini as described previously (3). The *C*-terminal Mtt protecting group was removed by washing the pre-swollen (3.5 h in

dichloromethane (DCM)) resin with a mixture of TFA/triisopropylsilane (TIS)/DCM (1:5:94, 3 x 2 min, then 4 x 5 min) until the filtrate was colorless. The resin was washed with DCM, methanol, DMF and then was swollen 2 h in NMP. The TFA salt was neutralized by washing the resin with 10 % DIEA in DMF (2 x) and then DMF. A solution of 3-maleimidopropionic acid (0.169 g, 1.00 mmol), *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU; 0.380 g, 1.00 mmol), and DIEA (349 μL, 2.00 mmol) was added and then shaken 2 h. The resin was washed with DMF, methanol, DCM, diethyl ether and then dried under high vacuum. The product was cleaved from the resin over 2 h in a mixture of TFA/TIS/H_2O (95:2.5:2.5), precipitated with diethyl ether, and then purified by HPLC (15 % B, hold 2 min then gradient to 90 % B, over 23 min) to afford (LLP2A)2-biotin-maleimide as a colorless solid (78 mg). HRMS (ESI) calculated for $C_{173}H_{256}N_{40}O_{42}S$: 3597.8841. Found 3597.8733.

SUPPLEMENTAL REFERENCES

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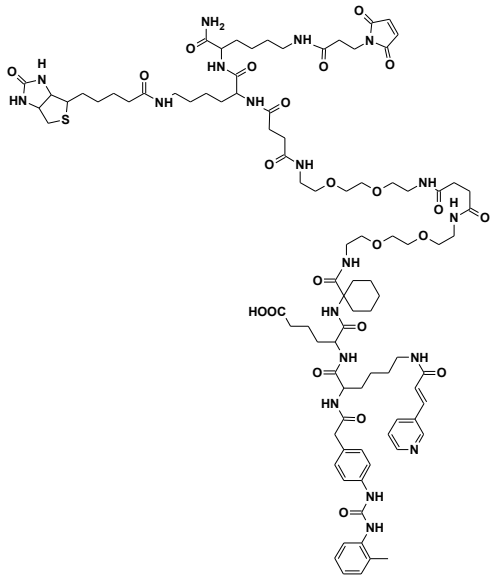
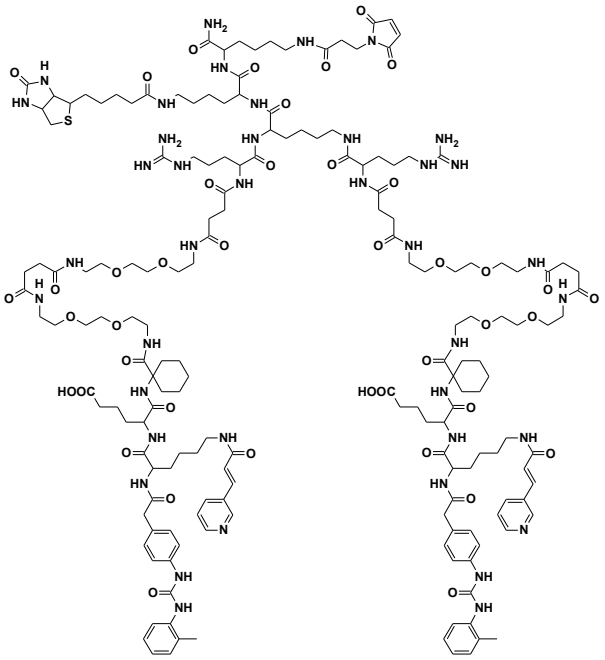
3. Song, A., Wang, X., Zhang, J., Marik, J., Lebrilla, C. B., and Lam, K. S. (2004) Synthesis of hydrophilic and flexible linkers for peptide derivatization in solid phase. *Bioorg. Med. Chem. Lett.* **14**, 161-165

SUPPLEMENTAL FIGURE

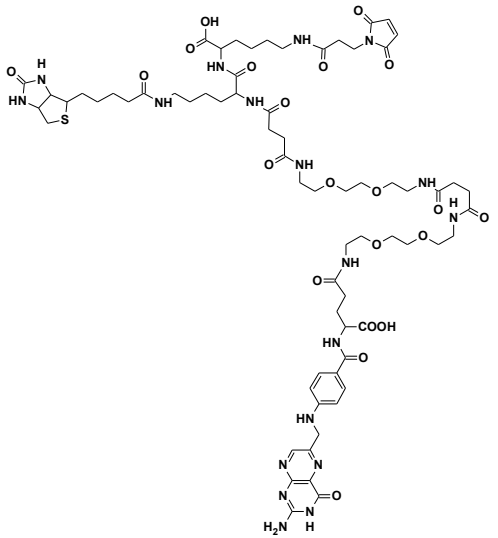
SUPPLEMENTAL FIGURE S1. Synthetic compounds for chemical programming. Structures of LLP2A-biotin-maleimide (*left*), (LLP2A)2-biotin-maleimide (*center*), and folate-biotin-maleimide (*right*).



LLP2A-biotin-maleimide



(LLP2A)2-biotin-maleimide



folate-biotin-maleimide

Fig. S1