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Letter

Incorporation of Fluorine into an OBOC Peptide Library by Copper-Free Click Chemistry toward the Discovery of PET Imaging Agents

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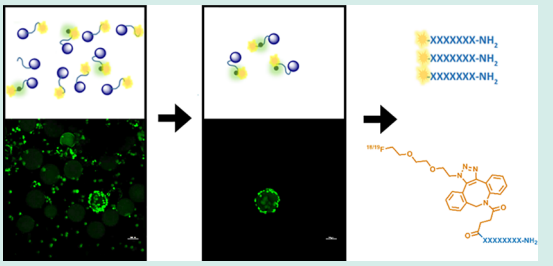
ABSTRACT: A one-bead one-compound (OBOC) library of peptide-based imaging agents was developed where a ^{19}F-containing moiety was added onto the N-terminus of octamer peptides through copper-free click chemistry prior to screening of the library. This created a library of complete imaging agents that was screened against CXCR4, a receptor of interest for cancer imaging. The screen directly resulted in the discovery of a peptide-based imaging agent with an IC_{50} of 138 μM. This proof-of-concept study describes a new type of OBOC peptide library design, where hits discovered from screening can be easily translated into their fluorine-18 counterpart for PET imaging without loss of affinity.



KEYWORDS: *OBOC, fluorine-18, click chemistry, molecular imaging, CXCR4*

New peptide-based imaging agents can be developed by rational design from known target-specific peptides or through combinatorial methods. The synthesis of one-bead one-compound (OBOC) libraries is a combinatorial method introduced by Lam et al. in 1991[1] that involves split-and-mix synthesis to create millions of candidates from which peptide–receptor interactions can be discovered. Although the screening of classic OBOC libraries produces target-specific peptides,[2−6] post-identification modification of these peptides to convert them into imaging agents often decreases binding affinity and creates the necessity for further modifications.[7−11] However, few ventures have been made into making OBOC libraries of imaging agents.[12−14]

Fluorine-18 is a preferred radioisotope for positron emission tomography (PET) imaging due to its half-life and availability; as such, incorporating a fluorine-19 isotopologue of a fluorine-18 prosthetic group into each entity of an OBOC library would create a library of complete potential imaging agents. This allows the portion of the imaging agent responsible for the imaging signal to be incorporated into the binding pocket of the target protein during affinity studies. Hereby, peptide sequences where this imaging portion would prevent binding would be excluded before further synthesis and complex binding assays. Conversely, peptides that bind better with the imaging portion present compared to the unmodified peptide are now included in further studies. This enables high-throughput screening of millions of potential imaging agents. Tang et al. recently created a focused OBOC peptide library containing both the $\alpha_v\beta_6$-targeting motif and a C-terminal 4-fluorobenzoyl group for imaging and had success in discovering lead peptides toward developing new ^{18}F PET agents.[14]

In this work, we aimed to develop an entirely randomized OBOC library containing an N-terminal functionalized fluoride-containing moiety in hopes of producing imaging agents where the portion containing the imaging isotope would be involved in receptor-binding itself. Copper-free click chemistry by strain-promoted alkyne–azide cycloaddition (SPAAC)[15] is an increasingly popular technique for incorporating radioisotopes into biomolecular targeting agents due to its quick and mild reaction conditions.[16−18] The design of the library was such that the eventual use of cyclooctyne-modified peptides and an azide-containing ^{18}F-prosthetic group would allow for efficient and simple radiolabeling to produce the ^{18}F-imaging agent for PET imaging.

The chemokine receptor CXCR4 has been implicated as a receptor of interest in a wide variety of cancers, especially in advanced and metastatic stages.[19] Current peptide-based ^{18}F PET imaging agents that target CXCR4 have been based on the CXCR4-antagonist peptide T140, which was developed from the natural peptide polyphemusin II.[20] Unfortunately, many of these imaging agents display poor pharmacokinetics and biodistribution in vivo.[21−24] New CXCR4-binding peptide-based imaging agents could be discovered through combinatorial methods.

Our unique combinatorial library was screened on-resin against a U87 cell line that highly expresses CXCR4; beads that showed binding to the cells were isolated, and the sequences of

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the respective peptide portions were identified by MALDI tandem mass spectrometry (MS/MS). This demonstrates a methodology where novel fluorine-containing peptide-based imaging agents can be discovered with straightforward translation into ^{18}F PET imaging agents for CXCR4-expressing cancer.

The cyclooctyne moiety chosen for the construction of our library was azadibenzocyclooctyne acid (ADIBO−COOH) **1**, which was synthesized according to the methods of Chadwick et al.[25] We chose to employ a PEG-based azide-containing ^{18}F-prosthetic group in order to offer some hydrophilicity to balance the large hydrophobic cyclooctyne. The fluorine-19 analogue **2** was synthesized and used in the construction of our library. An entirely randomized octamer OBOC library was synthesized on 1 g of Tentagel S resin equipped with a photocleavable linker ((3-amino-3-(2-nitro-phenyl)propionic acid) (ANP)). This allows protecting groups to be removed while the peptides stay attached to the resin beads; yet, the peptides can be later removed from the resin for sequencing. A total of 17 natural amino acids (excluding cysteine, methionine, and isoleucine) were used in the split-and-mix process, after which the cyclooctatriazole portion **3** was coupled onto the N-terminus of the combined library (Figure 1). The result was a pool of about 1.5 million potential imaging

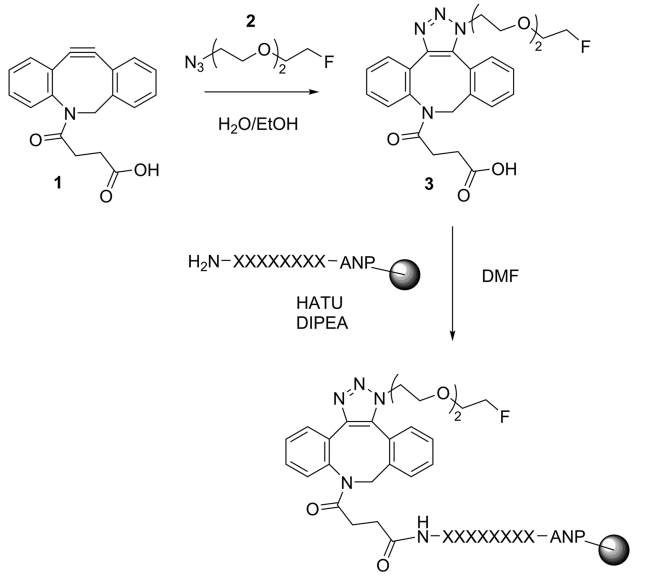

Figure 1. Synthesis of an octamer library on Tentagel resin using ADIBO−COOH **1** and a PEG-based azide-containing fluoride **2**. X is any natural amino acid excluding cysteine, methionine, and isoleucine. Only one regioisomer of the library constructs is shown for simplicity.

agent candidates, in which there is likely no redundancy of peptide sequences (less than 0.1% of potential diversity explored).

The deprotected library beads were subjected to screening against U87.CD4.CXCR4[26] cells that had been preincubated with a green CellTracker dye (ThermoFisher) to afford a way to visualize and sort the beads. The entire library was divided into multiple wells and screened; in each well, approximately 500 000 cells were incubated with 20 000 beads in 3 mL of media for 1 h at 37 °C. After incubation, some wells were visualized under fluorescence microscopy to confirm interactions between the cells and beads. Receptor−peptide interactions between the cells and beads were fixed with 4% paraformaldehyde, and excess paraformaldehyde was quenched with 2 M glycine. Library beads were then recombined, media was decanted, and the beads were resuspended in PBS.

Beads were sorted in aliquots using a Complex Object Parametric Analyzer and Sorter (COPAS, Union Biometrica)[27] through two screening steps. Initially, all beads were collected that expressed any threshold of green fluorescence (dye $\lambda_{ex/em}$ = 492/517 nm), which resulted in narrowing the library hits to approximately 2500 beads. This pool, however, can include false positive beads that exhibit autofluorescence of the Tentagel bead. These beads were then subjected to a second round of sorting by the COPAS with the green fluorescence threshold set to a higher limit (Figure 2). Hit beads during this

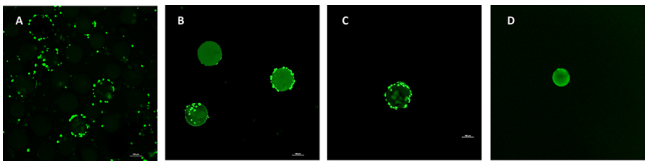

Figure 2. Confocal fluorescence images of (A) library pool after incubation with U87.CD4.CXCR4 cells tagged with CellTracker Green CMFDA; (B) library beads after initial screen showing some hit beads with various degrees of cell coverage; (C) isolated hit library bead in a well of a 96-well plate after second sorting round; (D) isolated false positive bead displaying high autofluorescence.

step were sorted individually into 96-well plates, and around 200 beads were collected. Visualization of the wells was performed under fluorescence microscopy to remove any remaining false positive beads that were incorrectly identified as hits during the automated COPAS sort due to high levels of bead autofluorescence (Figure 2).

Remaining beads were swollen in water and placed under a UV lamp for 2 h in order to cleave peptides off the beads. The supernatant was then subjected to MALDI MS/MS for peptide sequence deconvolution (Figure 3).

This step of the process proved challenging as some wells produced no observable MS product (possibly due to insolubility, incomplete cleavage from the bead, etc.) or incomplete fragmentation in MS/MS leading to unsolvable sequences (a known issue in this deconvolution technique).[28] In addition, the near isobaric amino acids lysine and glutamine were both used in this library to promote diversity, but the corresponding residue mass was often observed in MS/MS spectra, which lead to multiple possible iterations of the hit from a single bead. An unexpected issue that arose was the high frequency of fragmentation between the N-terminal amino acid and the cyclooctatriazole portion (at b_0), causing less intense fragmentation patterns along the rest of the peptide backbone. In the end, hit sequences that could be fully identified (Table S1) were resynthesized on Tentagel resin, including all possible combinations with Lys/Gln. Unsurprisingly, due to the negatively charged nature of the CXCR4-binding pocket,[29] many hit sequences contained multiple positively charged residues, which could result in nonspecific binding of these peptides to cells. Evaluation of each hit sequence using bulk beads and two cell lines that differentially express CXCR4 allowed for a final determination of peptide sequences legitimately showing interaction with CXCR4. Peptides on resin were incubated with a single sequence in individual wells with either fluorescently tagged U87.CXCR4.CD4 cells or U87.CD4 cells to serve as a control.

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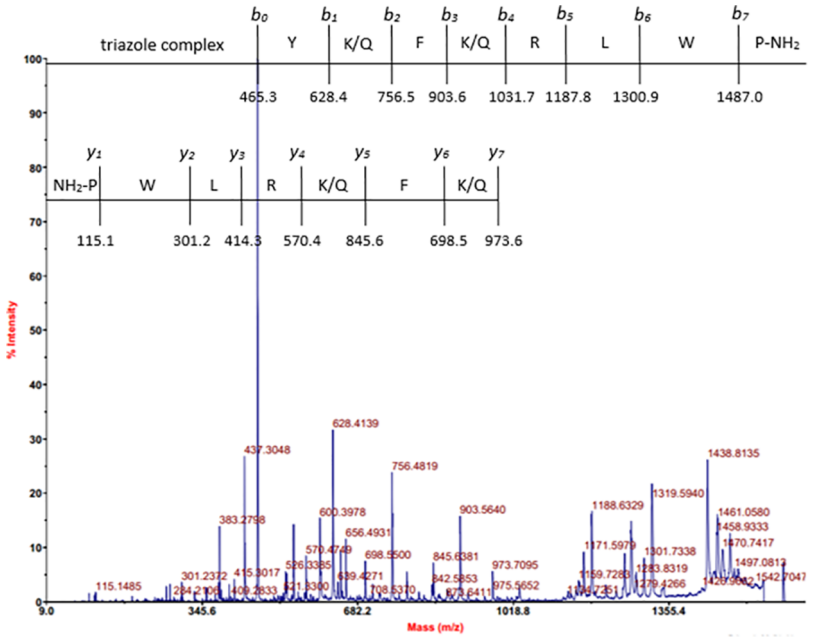

Figure 3. MALDI MS/MS deconvolution of a peptide hit from library screen, showing overlapping *b* and *y* series of fragments and demonstrating high fragmentation at b_0. The isobaric amino acids lysine (K) and glutamine (Q) are both possibilities for mass fragments of ∼128 Da.

Wells were imaged by fluorescent microscopy to reconfirm CXCR4 affinity and to select for peptides with CXCR4 selectivity. This step serves to remove any remaining false positives and to identify peptides that had nonspecific or off-target binding to the cells. Figure 4 displays an example where

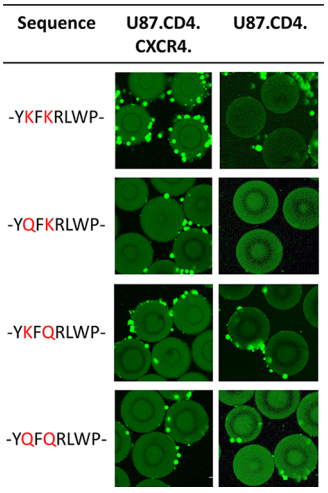

Figure 4. On-bead screen of potential hits synthesized on Tentagel of the sequence F-PEG_2-ADIBO-YXFXRLWP-NH_2 where X = Q or K. This screen allows for confirmation of "hit" sequence when isobaric amino acids are involved and also screens for selectivity to the target.

the four possible iterations of the "hit" sequence shown in Figure 2 were synthesized separately on Tentagel resin. Of the combinations, only one sequence (−YKFKRLWP−) showed good affinity to the CXCR4-expressing cells as well as strong selectivity for CXCR4 compared to the control cell line (cells in the top right image are in the background and not adherent to the beads). The other sequences show either low affinity, poor selectivity, or both.

CXCR4-selective peptides were then resynthesized on Rink Amide resin and purified as the C-terminal amide. These peptides were subjected to a competitive binding assay to prove receptor affinity using U87.CD4.CXCR4 cells and [^{125}I]-SDF-1α as the radioligand. The lead compound from this target affinity assay was identified as F-PEG_2-ADIBO-YKFKRLWP-NH_2 and has an IC_{50} of 138 μM. To determine whether the imaging moiety included within this peptide structure was essential for binding to CXCR4, we also performed a binding assay of the peptide H-YKFKRLWP-NH_2, which indicated that the unadorned peptide had the same binding affinity as the modified peptide (Figure S5). This suggests that the rather bulky portion containing the imaging component likely lies outside the binding pocket. While previously reported ^{18}F-peptide-based imaging agents for CXCR4 have higher affinities, in the nanomolar range, they are primarily based on the same polyphemusin peptide sequence and often have comparable drawbacks when it comes to in vivo behavior such as nonspecific binding to red blood cells and/or high liver and kidney uptake.[21−23] This OBOC library produced a new sequence that can serve as a starting point for further development toward novel CXCR4-targeted imaging agents.

Overall, we have developed an OBOC approach as a combinatorial method to produce a large-scale library of peptide imaging agent candidates. This can be useful for screening against any target of interest for imaging purposes. Our library conveniently includes fluorine-19 in place of our chosen imaging isotope fluorine-18 through copper-free click chemistry in order to produce hits that can be easily translated into radiolabeled imaging agents. Although the discovery of imaging agents from this method still proves to be laborious and fraught with challenges, the screening steps directly produced a lead imaging agent with micromolar affinity. Modifications based on this sequence could be explored to further improve the affinity for CXCR4. Exciting new advancements in technologies for tracking synthetic histories of solid-phase syntheses with DNA tags or RFID micro-transponders are highly complementary to this sort of application and will remove the sequencing bottleneck

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encountered, which would result in higher numbers of imaging agent hits to study.[30,31] Improvements in the methodology to address some of the challenges encountered in development of this library will be explored in future generations of OBOC imaging agent libraries. Focused library synthesis would aid in the throughput of sequence deconvolution when part of the sequence is known as well as likely produce higher affinity hits.[5,14] Other library conformations where the imaging moiety is dispersed through the peptide sequences could also be explored to promote discovery of imaging agents where the imaging moiety is integrated into the receptor-binding pocket.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.9b00146.

Synthetic procedures and characterization as well as further screening details and confocal microscopy images (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADIBO, azadibenzocyclooctyne; CXCR4, chemokine receptor 4; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry; OBOC, one-bead one-compound; PET, positron emission tomography; SPAAC, strain-promoted alkyne−azide cycloaddition

■ REFERENCES

(1) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. A New Type of Synthetic Peptide Library for Identifying Ligand-Binding Activity. *Nature* **1991**, *354*, 82−84.

(2) Yao, N.; Xiao, W.; Wang, X.; Marik, J.; Park, S. H.; Takada, Y.; Lam, K. S. Discovery of Targeting Ligands for Breast Cancer Cells Using the One-Bead One-Compound Combinatorial Method. *J. Med. Chem.* **2009**, *52* (1), 126−133.

(3) Komnatnyy, V. V.; Nielsen, T. E.; Qvortrup, K. Bead-Based Screening in Chemical Biology and Drug Discovery. *Chem. Commun.* **2018**, *54* (50), 6759−6771.

(4) Aina, O. H.; Liu, R.; Sutcliffe, J. L.; Marik, J.; Pan, C. X.; Lam, K. S. From Combinatorial Chemistry to Cancer-Targeting Peptides. *Mol. Pharmaceutics* **2007**, *4* (5), 631−651.

(5) Xiao, W.; Wang, Y.; Lau, E. Y.; Luo, J.; Yao, N.; Shi, C.; Meza, L.; Tseng, H.; Maeda, Y.; Kumaresan, P.; et al. The Use of One-Bead One-Compound Combinatorial Library Technology to Discover High-Affinity Avβ3 Integrin and Cancer Targeting Arginine-Glycine-Aspartic Acid Ligands with a Built-in Handle. *Mol. Cancer Ther.* **2010**, *9* (10), 2714−2723.

(6) Wang, W.; Wei, Z.; Zhang, D.; Ma, H.; Wang, Z.; Bu, X.; Li, M.; Geng, L.; Lausted, C.; Hood, L.; et al. Rapid Screening of Peptide Probes through In Situ Single-Bead Sequencing Microarray. *Anal. Chem.* **2014**, *86* (23), 11854−11859.

(7) Hu, L. Y.; Kelly, K. A.; Sutcliffe, J. L. High-Throughput Approaches to the Development of Molecular Imaging Agents. *Mol. Imaging Biol.* **2017**, *19* (2), 163−182.

(8) Gagnon, M. K. J.; Hausner, S. H.; Marik, J.; Abbey, C. K.; Marshall, J. F.; Sutcliffe, J. L. High-Throughput in Vivo Screening of Targeted Molecular Imaging Agents. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (42), 17904−17909.

(9) Xiao, W.; Li, T.; Bononi, F. C.; Lac, D.; Kekessie, I. A.; Liu, Y.; Sanchez, E.; Mazloom, A.; Ma, A.-H.; Lin, J.; et al. Discovery and Characterization of a High-Affinity and High-Specificity Peptide Ligand LXY30 for in Vivo Targeting of A3 Integrin-Expressing Human Tumors. *EJNMMI Res.* **2016**, *6* (1), 18.

(10) Peng, L.; Liu, R.; Marik, J.; Wang, X.; Takada, Y.; Lam, K. S. Combinatorial Chemistry Identifies High-Affinity Peptidomimetics against A4β1 Integrin for in Vivo Tumor Imaging. *Nat. Chem. Biol.* **2006**, *2* (7), 381−389.

(11) Beaino, W.; Nedrow, J. R.; Anderson, C. J. Evaluation of (68)Ga- and (177)Lu-DOTA-PEG4-LLP2A for VLA-4-Targeted PET Imaging and Treatment of Metastatic Melanoma. *Mol. Pharmaceutics* **2015**, *12* (6), 1929−1938.

(12) Cruickshank, D. R.; Luyt, L. G. The Development of Organometallic OBOC Peptide Libraries and Sequencing of N-Terminal Rhenium(I) Tricarbonyl-Containing Peptides Utilizing MALDI Tandem Mass Spectrometry. *Can. J. Chem.* **2015**, *93*, 234−243.

(13) Singh, J.; Lopes, D.; Gomika Udugamasooriya, D. Development of a Large Peptoid−DOTA Combinatorial Library. *Biopolymers* **2016**, *106* (5), 673−684.

(14) Tang, Y. S. C.; Davis, R. A.; Ganguly, T.; Sutcliffe, J. L. Identification, Characterization, and Optimization of Integrin α(v)B_6-Targeting Peptides from a One-Bead One-Compound (OBOC) Library: Towards the Development of Positron Emission Tomography (PET) Imaging Agents. *Molecules* **2019**, *24* (2), 309.

(15) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. A Strain-Promoted [3 + 2] Azide-Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *J. Am. Chem. Soc.* **2005**, *127* (31), 11196−11196.

(16) Meyer, J. P.; Adumeau, P.; Lewis, J. S.; Zeglis, B. M. Click Chemistry and Radiochemistry: The First 10 Years. *Bioconjugate Chem.* **2016**, *27* (12), 2791−2807.

(17) Campbell-Verduyn, L. S.; Mirfeizi, L.; Schoonen, A. K.; Dierckx, R. A.; Elsinga, P. H.; Feringa, B. L. Strain-Promoted Copper-Free "Click" Chemistry for 18F Radiolabeling of Bombesin. *Angew. Chem., Int. Ed.* **2011**, *50* (47), 11117−11120.

https://dx.doi.org/10.1021/acscombsci.9b00146
ACS Comb. Sci. XXXX, XXX, XXX−XXX

(18) Lim, S. T.; Kim, E.-M.; Jadhav, V. H.; Lee, S. B.; Jeong, H.-J.; Kim, D. W.; Sachin, K.; Kim, H. L.; Sohn, M.-H. F-18 Labeling Protocol of Peptides Based on Chemically Orthogonal Strain-Promoted Cycloaddition under Physiologically Friendly Reaction Conditions. *Bioconjugate Chem.* **2012**, *23* (8), 1680−1686.
(19) Balkwill, F. The Significance of Cancer Cell Expression of the Chemokine Receptor CXCR4. *Semin. Cancer Biol.* **2004**, *14* (3), 171−179.
(20) Tamamura, H.; Xu, Y.; Hattori, T.; Zhang, X.; Arakaki, R.; Kanbara, K.; Omagari, A.; Otaka, A.; Ibuka, T.; Yamamoto, N.; et al. A Low-Molecular-Weight Inhibitor against the Chemokine Receptor CXCR4: A Strong Anti-HIV Peptide T140. *Biochem. Biophys. Res. Commun.* **1998**, *253* (3), 877−882.
(21) Yan, X.; Niu, G.; Wang, Z.; Yang, X.; Kiesewetter, D. O.; Jacobson, O.; Shen, B.; Chen, X. Al[18F]NOTA-T140 Peptide for Noninvasive Visualization of CXCR4 Expression. *Mol. Imaging Biol.* **2016**, *18* (1), 135−142.
(22) Zhang, X.-X.; Sun, Z.; Guo, J.; Wang, Z.; Wu, C.; Niu, G.; Ma, Y.; Kiesewetter, D. O.; Chen, X. Comparison of 18F-Labeled CXCR4 Antagonist Peptides for PET Imaging of CXCR4 Expression. *Mol. Imaging Biol.* **2013**, *15* (6), 758−767.
(23) Jacobson, O.; Weiss, I. D.; Kiesewetter, D. O.; Farber, J. M.; Chen, X. PET of Tumor CXCR4 Expression with 4−18F-T140. *J. Nucl. Med.* **2010**, *51* (11), 1796−1804.
(24) Turnbull, W. L.; Yu, L.; Murrell, E.; Milne, M.; Charron, C. L.; Luyt, L. G. A Dual Modality 99m Tc/Re(i)-Labelled T140 Analogue for Imaging of CXCR4 Expression. *Org. Biomol. Chem.* **2019**, *17* (3), 598−608.
(25) Chadwick, R. C.; Van Gyzen, S.; Liogier, S.; Adronov, A. Scalable Synthesis of Strained Cyclooctyne Derivatives. *Synthesis* **2014**, *46* (5), 669−677.
(26) Björndal, A.; Deng, H.; Jansson, M.; Fiore, J. R.; Colognesi, C.; Karlsson, A.; Albert, J.; Scarlatti, G.; Littman, D. R.; Fenyö, E. M. Coreceptor Usage of Primary Human Immunodeficiency Virus Type 1 Isolates Varies According to Biological Phenotype. *J. Virol.* **1997**, *71* (10), 7478−7487.
(27) Cho, C. F.; Behnam Azad, B.; Luyt, L. G.; Lewis, J. D. High-Throughput Screening of One-Bead-One-Compound Peptide Libraries Using Intact Cells. *ACS Comb. Sci.* **2013**, *15* (8), 393−400.
(28) Medzihradszky, K. F.; Chalkley, R. J. Lessons in de Novo Peptide Sequencing by Tandem Mass Spectrometry. *Mass Spectrom. Rev.* **2015**, *34* (1), 43−63.
(29) Rosenkilde, M. M.; Gerlach, L.-O.; Jakobsen, J. S.; Skerlj, R. T.; Bridger, G. J.; Schwartz, T. W. Molecular Mechanism of AMD3100 Antagonism in the CXCR4 Receptor: Transfer of Binding Site to the CXCR3 Receptor. *J. Biol. Chem.* **2004**, *279* (4), 3033−3041.
(30) MacConnell, A. B.; McEnaney, P. J.; Cavett, V. J.; Paegel, B. M. DNA-Encoded Solid-Phase Synthesis: Encoding Language Design and Complex Oligomer Library Synthesis. *ACS Comb. Sci.* **2015**, *17* (9), 518−534.
(31) Vastl, J.; Wang, T.; Trinh, T. B.; Spiegel, D. A. Encoded Silicon-Chip-Based Platform for Combinatorial Synthesis and Screening. *ACS Comb. Sci.* **2017**, *19* (4), 255−261.

https://dx.doi.org/10.1021/acscombsci.9b00146
ACS Comb. Sci. XXXX, XXX, XXX−XXX