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DNA-Compatible Conditions for the Formation of *N*-Methyl Peptide Bonds

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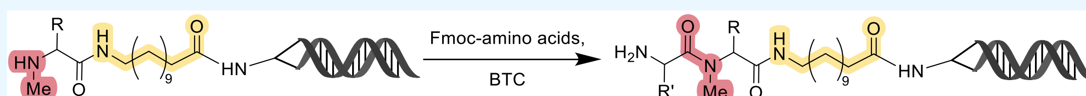

ABSTRACT: DNA-encoded libraries (DELs) are a powerful platform in drug discovery. Peptides have unique properties that make them attractive pharmaceutical candidates. N-methylation of the peptide backbone can confer beneficial properties such as increased proteolytic stability and membrane permeability. Herein, we evaluate different DEL reaction systems and report a DNA-compatible protocol for forming N-methylated amide bonds. The DNA-compatible, bis(trichloromethyl)carbonate-mediated amide coupling is efficient for the formation of N-methyl peptide bonds, which promises to increase the opportunity to identify passively cell-permeable macrocyclic peptide hits by DNA-encoded technology.

INTRODUCTION

DNA-encoded libraries (DELs) are powerful tools for lead identification, enabling the testing of unprecedented numbers of compounds for binding to proteins of pharmaceutical interest.[1−3] The efficiency and generality of DNA-compatible amide bond formation and the availability of a large panel of building blocks make macrocyclic peptides attractive targets for large DEL campaigns.[1] Macrocyclic peptides have the potential to penetrate cell membranes and target protein−protein interactions.[4,5] Cyclosporine A, a successful macrocyclic peptide drug with good membrane permeability, contains 11 amino acids, seven of which are N-methylated. Previous structure-permeability studies have demonstrated that N-methylation can improve the membrane permeability of macrocyclic peptides.[4,6,7] However, incorporating N-methyl amino acids into DEL peptides is challenging due to the steric hindrance caused by the secondary amine, which under typical solid phase peptide synthesis (SPPS) conditions often requires harsh conditions and multiple couplings. A study of peptide bond formation by Franizi et al. revealed that secondary amines on DNA provided constantly lower yield in the amide bond formation.[8]

The wealth of coupling reagents available for use in traditional SPPS has not been systematically evaluated in the preparation of DNA-encoded peptide libraries. For instance, bis(trichloromethyl) carbonate (BTC, a.k.a. triphosgene), which is a highly efficient reagent for forming N-methyl amide bonds in SPPS, has not been investigated for N-methyl amide coupling on DNA.[9] Acyl chlorides have been explored as highly active species for hindered amide couplings. Wrenn et al. used trichloroacetonitrile for acid chloride formation, which allowed the coupling of Fmoc-proline to hindered on-DNA peptoids.[10] We hypothesized that expanding the toolkit of available coupling protocols for DEL peptide synthesis could provide an opportunity to achieve the incorporation of N-methyl amino acids into DELs.

Efforts have been made to bridge the gap between DEL-compatible reactions and organic synthesis in order to expand the chemical space available to DNA-encoded molecules. Harbury,[11] Dawson,[12] and Berst[13] et al. reported the employment of ion-exchange resins as reversible solid supports to perform chemical transformations on immobilized DNA conjugates under anhydrous conditions. Additionally, micelles offer alternative reaction media for DEL chemistry.[14−20] Waring et al. developed an efficient and applicable method for DEL amide reactions under micellar conditions.[19] Despite the continuing evolution of DNA-compatible chemistries, however, the coupling of protected amino acids onto DNA-conjugated N-methyl amines has thus far not been reported.

In our initial investigations, we found that DMT-MM and EDC/HOAt, two of the most commonly used coupling reagents for effecting amide bond formation in DELs,[21−23] failed to achieve efficient conversions in the coupling of Fmoc-amino acids onto DNA-conjugated peptides that terminate in N-methyl amines (Table S1). This prompted us to evaluate alternative reaction media for effecting more challenging amide couplings, including ion-exchange resins, micellar conditions, and cosolvent systems. We screened various coupling reagents for DEL amide coupling and found that, despite some limitations in the reaction scope, BTC is a promising reagent

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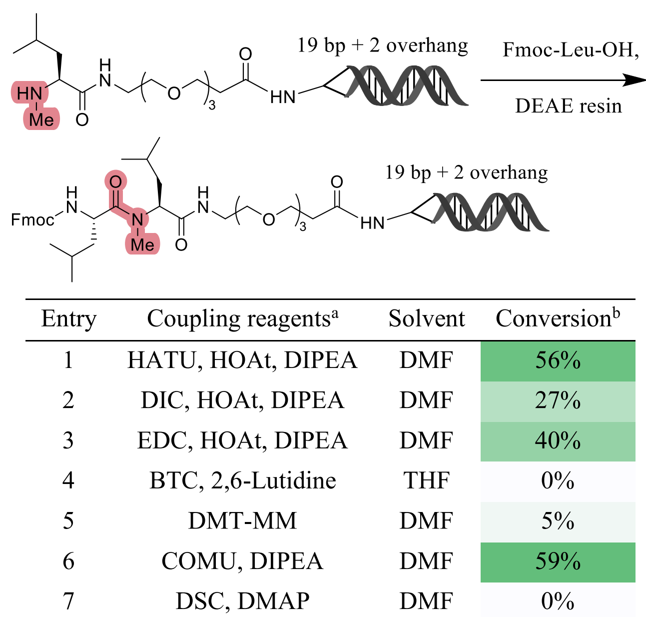
for achieving high coupling efficiency to form N-methyl peptide bonds on DNA.

■ RESULTS AND DISCUSSION

Resin-Adsorbed DEL Amide Coupling. A promising tool for effecting organic reactions on DNA-conjugated substrates is to use solid supports such as ion-exchange resins to reversibly capture the DNA from the reaction mixture. This approach can enable reactions of DELs in organic solvents and has been used to effect organic reactions such as decarboxylative cross-coupling,[12,13] electrochemical amination,[12] saturated heterocycle synthesis,[13] reductive amination,[12] sulfide and sulfonamide formation,[24] and traditional peptide couplings.[11,12] Several ion-exchange resins have been reported for this application.[11−13,24] Here, we selected two resins that have been reported for DEL amide couplings: DEAE Sepharose resin[11] and Phenomenex Strata-XA resin.[12]

In the initial screening of the two resins, we performed a simple amide coupling onto a non-N-methylated peptide conjugated to short (8-mer) dsDNA (Table S2). The DEAE Sepharose resin achieved higher conversion and recovery on the initial screening of the test amide coupling. In contrast, the Strata-XA resin resulted in lower coupling efficiency and recovery of DNA for the test reaction (Table S2). Therefore, we explored the DEAE resin further by screening seven coupling reagents for the ability to couple onto a Leu(NMe) amine conjugated to a longer (21mer) DNA strand (Table 1). HATU (Table 1, **Entry 1**) and COMU (Table 1, **Entry 6**) achieved moderate conversions, with the appearance of side products corresponding to multiple additions of Fmoc-Leu-OH to the DNA (Figure S2). We speculated that the amino acid coupled not only to the expected peptide position but also to the nucleotides of the DNA tag. To test whether it was the length of the DNA or the more challenging N-Me coupling that led to this decrease in efficiency, we investigated the coupling of Fmoc-Leu-OH onto an N-methyl amine conjugated to a shorter 8-mer dsDNA adsorbed onto DEAE Sepharose (Figure S3). This coupling generated 85% product with the desired N-methyl amide bond with no acylation side product observed by LC-MS (Figure S3). Therefore, the quality of DEL amide coupling on the DEAE Sepharose resin appears to decrease as the dsDNA tag increases in length. Since the DNA tags in a typical DEL synthesis often reach well beyond 50 nucleotides, resin-adsorbed amide couplings may not be suitable for DELs requiring the synthesis of peptides with N-methyl backbone amides.

Table 1. Conversion of DEAE Resin-Adsorbed DEL Amide Couplings



Entry	Coupling reagents[a]	Solvent	Conversion[b]
1	HATU, HOAt, DIPEA	DMF	56%
2	DIC, HOAt, DIPEA	DMF	27%
3	EDC, HOAt, DIPEA	DMF	40%
4	BTC, 2,6-Lutidine	THF	0%
5	DMT-MM	DMF	5%
6	COMU, DIPEA	DMF	59%
7	DSC, DMAP	DMF	0%

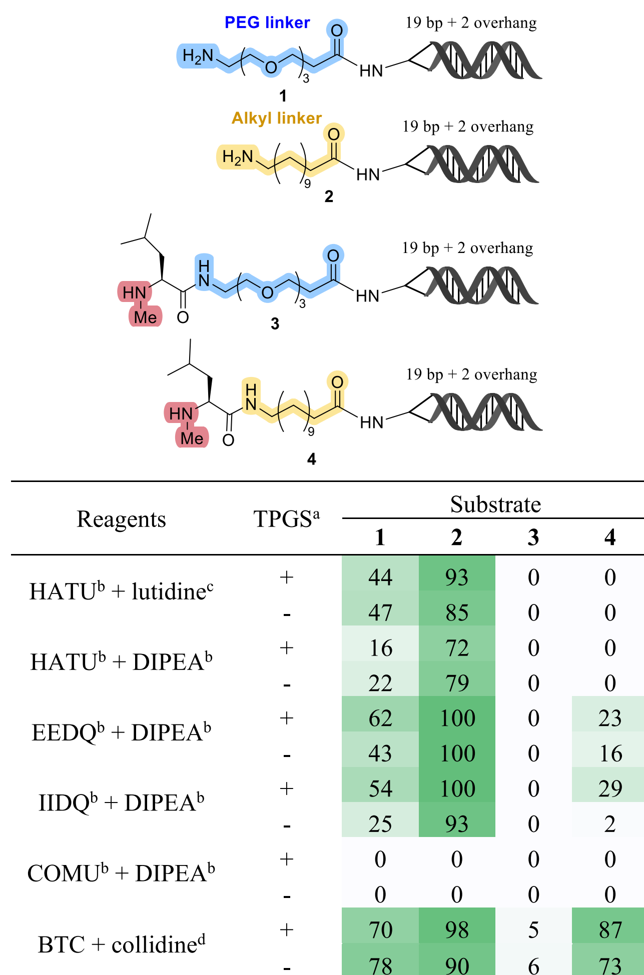
Deeper green corresponds to a higher conversion. [a]Concentrations of reagents in the final reaction solution: Fmoc-Leu-OH (75 mM), coupling reagent (50 mM), and base (150 mM). [b]Conversion includes side products. Side products were overlapped with products in UV chromatography but identified by the mass spectrum.

Micellar DEL Amide Coupling. The micelles generated by surfactants dissolved in water offer an alternative reaction medium for DEL chemistry.[14−20] Micelles can concentrate hydrophobic reagents and substrates into their hydrocarbon cores, thus physically separating the chemistry taking place at the DEL's reactive end from the DNA.[18] The neutral surfactant TPGS-750-M has been studied in depth for its ability to facilitate DEL chemistry, although TPGS-750-M had not been investigated in the coupling of Fmoc-amino acids to N-methyl amine nucleophiles.[14−16,20]

We synthesized four DNA-peptide conjugates (**1**−**4**) to investigate micellar conditions for the formation of N-methyl amide bonds on DELs. Conjugates **1** and **2** terminate in a simple primary amine, and conjugates **3** and **4** terminate in the secondary amine of NH(Me)-Leu. Waring et al. reported that an alkyl linker could significantly improve reaction efficiency under micellar conditions, while a PEG linker was thought to disfavor the association with the surfactant.[19] Therefore, we constructed the conjugates with either a PEG linker (**1** and **3**) or an alkyl linker (**2** and **4**). Acylations were initially performed with the model substrate Fmoc-Leu-OH using a variety of different coupling reagents (Table 2). The percentage of surfactant (TPGS-750-M), temperature, and incubation time was based on literature procedures optimized for micellar amide couplings.[19] To explore the surfactant effect, reactions without the surfactant were performed in deionized water. The data in Table 2 underscores the well-known challenge of coupling amino acids to secondary (**3** and **4**) compared to primary (**1** and **2**) amines. Consistent with the observations by Waring et al.,[19] the linker had a strong effect on coupling efficiency, with much higher conversions observed for the alkyl linker compared to the PEG linker (Table 2). Taken together, these results suggest that the alkyl linker alone may induce a phase-separated microenvironment in which water is excluded and/or the active ester is concentrated near the reactive amine.

Among screened coupling reagents, EEDQ and IIDQ functioned better in the micellar conditions and gave clean desired products with high conversions using model primary amine **2**. However, couplings onto the more hindered amine of Leu(NMe) (**4**) using EEDQ/IIDQ led to significant capping of the amine to form the ethyl or isobutyl carbamate, respectively (Figures S8 and S9). We also performed coupling reactions between **2**/**4** and two other amino acids, Fmoc-Gly-OH and Fmoc-Ala-OH, using EEDQ or IIDQ as coupling reagents. The corresponding carbamate side products were also observed in the reactions with **4** (Tables S3 and S4). The secondary amine, with its higher nucleophilicity, might be more predisposed to form the carbamate with EEDQ or IIDQ. BTC was most effective in forming an N-methyl amide bond,

Table 2. Screening Coupling Reagents for the Couplings of Fmoc-Leu-OH to Various Substrates and Linkers under Micellar Conditions



Reagents	TPGS[a]	Substrate 1	2	3	4
HATU[b] + lutidine[c]	+	44	93	0	0
	-	47	85	0	0
HATU[b] + DIPEA[b]	+	16	72	0	0
	-	22	79	0	0
EEDQ[b] + DIPEA[b]	+	62	100	0	23
	-	43	100	0	16
IIDQ[b] + DIPEA[b]	+	54	100	0	29
	-	25	93	0	2
COMU[b] + DIPEA[b]	+	0	0	0	0
	-	0	0	0	0
BTC + collidine[d]	+	70	98	5	87
	-	78	90	6	73

Percent product was shown in each cell. [a]3% TPGS-750-M solution (+) vs deionized water (−). Final concentration: Fmoc-Leu-OH (0.5 M). [b]0.5 M. [c]2 M. [d]Fmoc-Leu-OH, BTC, and 2,4,6-collidine were added to THF to give final concentrations of 0.5 M, 0.16 mM, and 1 M, respectively. After 10 min, this pre-activated solution was centrifuged, 20 μL of the supernatant was pipetted into a new tube, and THF was dried by air flow. Then, 30 μL of borate buffer containing the DNA conjugate (0.1 mM) and DIPEA (0.3 M) was added to the reaction. The reaction was performed at room temperature for 2 h.

resulting in more than 70% conversion on conjugate **4** in both water and micellar solution (Table 2). Complementary to Franzini's study on the amide couplings to sterically hindered amines on DNA,[8] BTC could be an alternative coupling reagent for DEL amide coupling to generate amide bonds more efficiently and with higher chemoselectivity.

Screening Coupling Reagents in Cosolvent Systems. In the investigation of DEL amide couplings in the presence of the surfactant, we found that couplings proceeded with similar efficiencies in water and micellar solution (Table 2), which prompted us to further assess the reproducibility of DEL peptide couplings in aqueous solution. We repeated the coupling between Fmoc-Leu-OH and the four DNA conjugates (**1**−**4**) in mixed solvents containing water and an organic cosolvent (Table 3). HATU in the cosolvent system

Table 3. Screening Coupling Reagents for the Couplings of Fmoc-Leu-OH to Various Substrates and Linkers under Cosolvent Systems

Reagents	Substrate 1	2	3	4
HATU + DIPEA[a]	100	92	0	0
EEDQ + DIPEA[a]	0	0	0	0
IIDQ + DIPEA[a]	0	0	0	0
COMU + DIPEA[a]	98	98	0	0
BTC + 2,4,6-collidine[b, c]	87	99	37	100

Percent product was shown in each cell. [a]DMF. [b]THF was used as a cosolvent. [c]Fmoc-amino acids, BTC, and 2,4,6-collidine were added to THF to give final concentrations of 0.5 M, 0.16 mM, and 1 M, respectively. After 10 min, the pre-activated solution was centrifuged. 20 μL of the supernatant was pipetted into 10 μL of DNA conjugate solution (0.3 mM in borate buffer). Then, 1.57 μL of DIPEA was added to give a final concentration of 0.3 M. The reaction was performed at room temperature for 2 h.

has been successfully applied for constructing DEL peptides.[25] Our data further confirmed that HATU is an efficient coupling reagent for the acylation of primary amines. However, we were not able to observe acylation to the DNA-conjugated secondary amine (Table 3). COMU performed better in the aqueous conditions with a DMF cosolvent compared to the micellar conditions. EEDQ and IIDQ were unable to facilitate the DEL amide couplings in the cosolvent system. We tried both DMF and THF as the cosolvent for BTC-mediated couplings, and THF provides higher coupling efficiencies. BTC-mediated coupling of conjugate **4** in THF achieved 100% conversion (Table 3). Under identical conditions, nucleophiles linked to DNA through PEG (**1** and **3**) were acylated with lower efficiency compared to the nucleophiles tethered via the all-alkyl linker (**2** and **4**) (Table 3). Similar observations were found in the micellar conditions, further indicating that the alkyl linker provides a microenvironment that favors amide bond formation, while the PEG linker disfavors acylation.

To better compare the micellar and cosolvent conditions for BTC-mediated couplings, we investigated the coupling of **4** with six hydrophobic amino acids (Table 4). The reactions generally showed higher conversions in the cosolvent condition, except for the coupling of **4** with Fmoc-(NMe)-Val-OH, which fared slightly better in micelles. The data revealed that the micellar and cosolvent conditions provide similar efficiencies in the BTC-mediated coupling onto a DNA-conjugated secondary amine nucleophile, with the cosolvent system performing slightly better than the micelles overall.

Reaction Condition Exploration. To further optimize these reaction conditions, we coupled Fmoc-Leu-OH onto the HN(Me)-Leu-DNA amine while varying the concentrations of reagents and the ratio of THF and water (Table 5). The reaction efficiency decreased with decreasing concentration of reactants (Table 5, **Entry 1**−**4**). We also tested lower concentrations of amino acids and coupling reagents under micellar conditions, which also gave lower coupling efficiencies (Table S5). The addition of DIPEA to the reaction significantly increased the conversion (Table 5, **Entry 1** vs **5**) while reducing the ratio of THF to water from 2:1 to 1:1 decreased conversion (Table 5, **Entry 1** vs **6**). In conclusion, the coupling efficiency benefits from a high concentration of reactants, a higher ratio of THF, and the presence of DIPEA.

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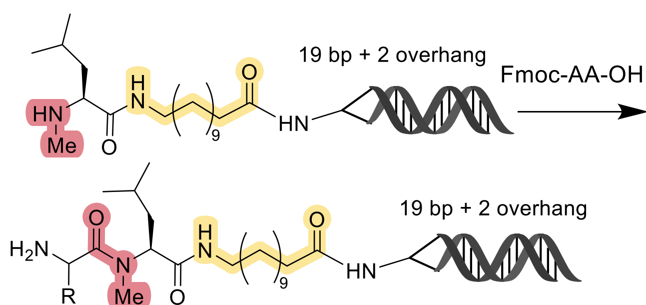
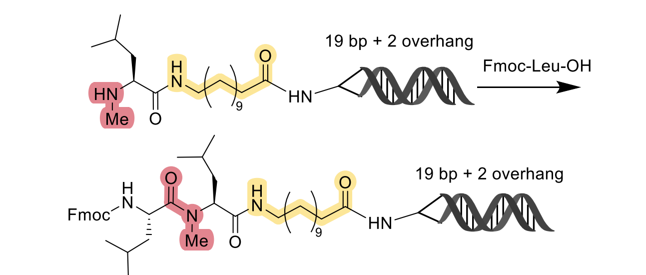
Table 4. Comparison of BTC-Mediated DEL Amide Couplings in Micellar Conditions and Cosolvent Conditions



Fmoc-AA-OH	condition	
	cosolvent system[a]	micellar condition[b]
Leu	100	87
Pro	92	87
Val	66	60
D-Phe	32	20
(NMe)Leu	67	63
(NMe)Val	63	74

% Product was shown in each cell. The coupling protocol was identical to the one shown in Tables 3[a] and 2[b].

Table 5. Condition Optimization of BTC-Mediated DEL Amide Couplings



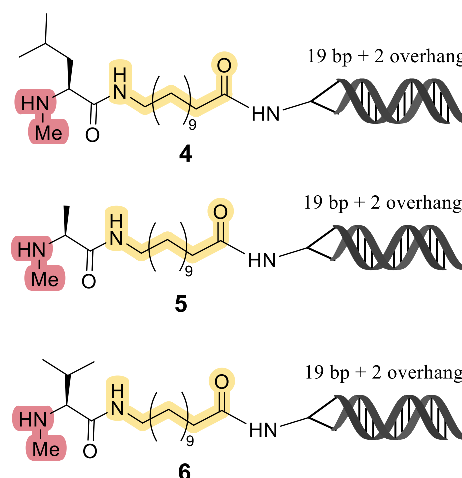
Entry	Pre-activation in THF (mM)[a]			DIPEA (mM)	THF: Borate buffer	% Product
	Fmoc-Leu-OH	BTC	Collidine			
1	500	167	1000	300	2:1	100
2	250	83	750	300	2:1	94
3	125	42	500	300	2:1	88
4	80	27	250	300	2:1	10
5	500	167	1000	0	2:1	40
6	500	167	1000	300	1:1	80

% Product was shown in each cell. Deeper green corresponds to higher conversion. [a]To a reaction tube containing THF was added Fmoc-Leu-OH, BTC, and collidine to give final concentrations as listed in the table. After 10 min, the pre-activated solution was centrifuged. 20 μL of the supernatant was pipetted into 10 μL of DNA conjugate solution with/without DIPEA. The reaction was performed at room temperature for 2 h.

Reaction Scope using BTC as Coupling Reagents. Next, we explored the substrate scope of BTC-mediated amide coupling. We performed an expanded-scope study on a combinatorial matrix between different DNA-peptide conjugates and Fmoc-amino acids (Tables 6 and S6).

Pleasingly, test reactions between a range of substrates and N-methyl amine conjugates (**4**–**6**) proceed in moderate to

Table 6. Reactions Scope of BTC-Mediated DEL Amide Couplings



Fmoc amino acids	4	5	6
Fmoc-Leu-OH	100	84	93[a]
Fmoc-D-Leu-OH	95	91	66
Fmoc-Pro-OH	92	91	75
Fmoc-Phe-OH	100	77[b]	65
Fmoc-D-Phe-OH	100[b]	43[b]	70
Fmoc-Val-OH	66[c]	64[c]	36[b, c]
Fmoc-NMe-Leu-OH	96[a]	77[a]	69[a]
Fmoc-NMe-Val-OH	68[a]	78[a]	3[a]
Fmoc-Sar-OH	14[b]	58[b]	76[a]
Fmoc-Gly-OH	0 (88[d])	42[b] (100[d])	0 (39[d])
Fmoc-Ala-OH	0 (43[d])	0 (61[d])	0 (0[d])
Fmoc-Abu-OH	0 (44[d])	0 (43[d])	0 (0[d])
Fmoc-Ser(Me)-OH	78	82	36
Fmoc-homePhe-OH	44[a]	43[a]	0
Fmoc-4-Methoxy-Phe-OH	90	54	56
Fmoc-D-beta-homoPhe-OH	88	88	72
Fmoc-Nva-OH	100	81	72
Fmoc-Met-OH	76	36	17
Fmoc-Asp(Ome)-OH	75	52	19
Fmoc-D-Glu-OtBu-OH	60	72	30
Fmoc-Trp-OH	13	13	14
Fmoc-Lys(Boc)-OH	37	30	6
Fmoc-D-Cha-OH	100[a]	90[a]	84[a]

% Product was shown in each cell. Deeper green corresponds to higher conversion. The conversion was improved by [a]double or [b]triple couplings (the conversion of the single couplings is shown in Table S6). [c]Side product was observed. [d]Conversion was obtained from double couplings using DMT-MM as the coupling reagent.

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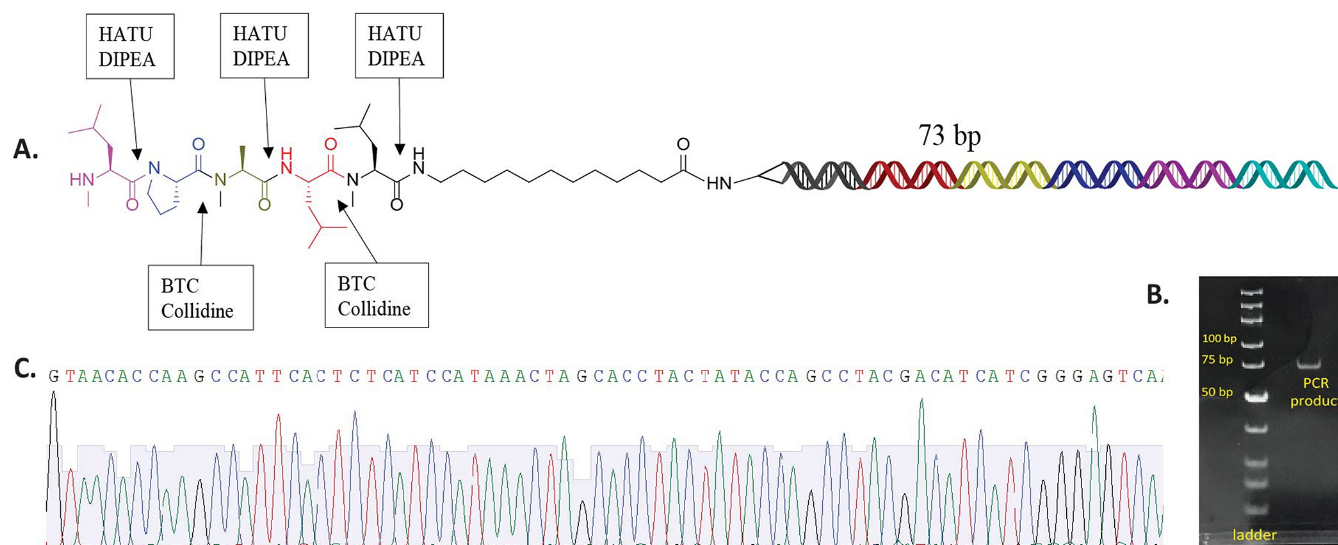

Figure 1. Test compound. (A) Structure and synthesis of the test compound. (B) Gel electrophoresis of the PCR product of the test compound. (C) Sanger sequencing of the PCR product.

excellent conversion to generate a clean product (Table 6). Even very difficult couplings, such as between Fmoc-Leu-(NMe)-OH and the secondary amine of Leu(NMe) conjugated to DNA, proceeded in a very high yield. The coupling of Fmoc-Val-OH generated a significant amount of side products corresponding to the addition of a second equivalent of Fmoc-Val-OH (Figure S25). The prevalence of this side product increased with additional couplings and could not be removed by treatment with 40% ammonium hydroxide and methyl amine (ν: ν = 1:1),[26] suggesting that it was the result of an irreversible acylation of the nucleobases of the encoding DNA. Despite the well-known challenges of coupling onto highly hindered, β-branched secondary amine nucleophile,[27] coupling onto Val(NMe) (**6**) was successful for a variety of Fmoc-amino acids, although the yields were somewhat lower compared to couplings onto less hindered substrates **4** and **5**.

We found that Fmoc-Abu-OH, Fmoc-Gly-OH, and Fmoc-Ala-OH coupled with quite poor efficiencies, perhaps due to the competition between coupling and hydrolysis of the active ester for these less hindered reactants. To improve the couplings of those three amino acids onto DNA-tethered NMe-amino acids, six coupling reagents were screened (Table S7). Among the reagents tested, DMT-MM showed the most promise. Fmoc-Gly-OH achieved higher conversions using DMT-MM compared to Fmoc-Ala-OH and Fmoc-Abu-OH (Table 6). DMT-MM was less effective for the couplings onto NMe-Val-DNA (Table 6).

We also performed these couplings on the counterparts of DNA conjugates with a PEG linker (Table S6). Consistent with the result of Table 5, the PEG linker caused very poor coupling efficiencies across. Therefore, the alkyl linker is required for achieving efficient couplings to N-methyl amines on DNA with a broad substrate scope. Given the remarkably efficient reaction profiles, the BTC-mediated protocol might find widespread use in the field of DNA-encoded library synthesis.

Synthesis of a Test Compound. To demonstrate that the developed BTC-mediated protocol is DNA-compatible, we synthesized a linear DNA-peptide conjugate and evaluated the integrity of the post-reaction DNA (Figure 1**A**). In each cycle, DNA ligation was performed, followed by the amide coupling either using HATU or BTC as a coupling reagent (Scheme S1). A closing tag was put onto the conjugate in the last step to give a 73 base-paired DNA-peptide conjugate (Figure S81). The post-reaction DNA was amplified by PCR, which gave a clean amplified product with the correct length (Figure 1B). The PCR product was subjected to Sanger sequencing, which produced the expected DNA sequence (Figure 1C). The post-reaction DNA on the test compound was amplified and quantified by qPCR. The amount of intact, amplifiable DNA was 92.4% of the total DNA input, indicating that the DNA maintained its integrity after multiple BTC-mediated peptide couplings. (Table S12). Cumulatively, the gathered data indicates that the BTC-mediated coupling condition is compatible with DNA over multiple coupling and DNA ligation cycles.

■ CONCLUSIONS

We evaluated different conditions for the acylation of DNA-conjugated secondary amino acids, including ion-exchange resins, micellar conditions, and cosolvent systems. We found that acylation on the bases of the encoding DNA might take place in the couplings on DEAE Sepharose resins under organic conditions. BTC is a suitable coupling reagent for forming N-methyl amide bonds on DNA. While we found that the commonly used surfactant TPGS-750-M had little effect on reaction efficiency, micellar conditions may be required based on the large difference in yield between alkyl- vs. PEG-tethered amines. Herein, we demonstrated a method to achieve the formation of N-methyl amide bonds on DEL peptides, thus expanding the DEL-accessible chemical space to include this important peptide backbone modification. The DNA-compatible BTC-mediated protocol has been shown to be highly effective for the formation of N-methyl peptide bonds with a relatively wide substrate scope and causes no apparent signs of DNA damage. However, our study indicates that certain amino acids could be out of the substrate scope of the developed protocol. Therefore, building block validation is recommended when the BTC-mediated protocol is used for DEL constructions. This work expands the toolkit for DEL reactions

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and contributes to a higher chance of discovering passive cell-permeable peptide hits using DNA-encoded technology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00576.

Chemical abbreviations, experimental methods, additional discussion, figures, schemes, tables, and LC-MS data (PDF)

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Author Contributions

S.L. and P.Z. conceived the research. P.Z. designed and performed the screening of different coupling reagents in different coupling conditions and synthesized the test compound. G.K. designed and performed the amide couplings using DMT-MM as the coupling reagent. Y.Z. conducted the substrate scope study of BTC-mediated couplings. K.Y. assisted with the TA clone for Sanger sequencing. P.Z. wrote the manuscript, and S.L. assisted with editing and revisions. All authors gave approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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