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Research Article

# Solution-Phase Fmoc-Based Peptide Synthesis for DNA-Encoded Chemical Libraries: Reaction Conditions, Protecting Group Strategies, and Pitfalls

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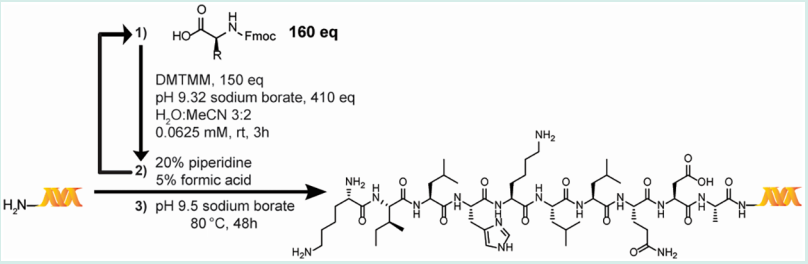


**ABSTRACT:** Peptide drug discovery has shown a resurgence since 2000, bringing 28 non-insulin therapeutics to the market compared to 56 since its first peptide drug, insulin, in 1923. While the main method of discovery has been biological display—phage, mRNA, and ribosome—the synthetic limitations of biological systems has restricted the depth of exploration of peptide chemical space. In contrast, DNA-encoded chemistry offers the synergy of large numbers and ribosome-independent synthetic flexibility for the fast and deeper exploration of the same space. Hence, as a bridge to building DNA-encoded chemical libraries (DECLs) of peptides, we have developed substrate-tolerant amide coupling reaction conditions for amino acid monomers, performed a coupling screen to illustrate such tolerance, developed protecting group strategies for relevant amino acids and reported the limitations thereof, developed a strategy for the coupling of $\alpha,\alpha$-disubstituted alkenyl amino acids relevant to all-hydrocarbon stapled peptide drug discovery, developed reaction conditions for the coupling of tripeptides likely to be used in DECL builds, and synthesized a fully deprotected DNA-decamer conjugate to illustrate the potency of the developed methodology for on-DNA peptide synthesis.

**KEYWORDS:** *aqueous Fmoc-based peptide synthesis, all-hydrocarbon stapled peptides, peptide drug discovery*

## INTRODUCTION

In living organisms, peptides act as short-lived, cell-impermeable endocrine signaling molecules that are essential for normal function. Peptide drugs, such as insulin have had a significant impact on thetherapeutic landscape.[1] Alternatively, peptides are attractive candidates for the intractable intracellular protein−protein interactions (PPIs) that comprise the majority of the targetome.[2] Falling into a molecular class between small molecules and proteins,[3] peptides have indeed proven to be potent binders[4−6] of the shallow, featureless surfaces of PPIs.[7,8] Long marginalized, peptide drug discovery has shown a resurgence since 2000, providing 28 non-insulin peptide drugs worldwide. This represents half the total number of approved peptide therapeutics since the commercialization of the first one, insulin, in 1923.[1] Concentrated in metabolic diseases and oncology, the market share of peptide drugs is predicted to rise as those therapeutic areas unfortunately claim more patients in the years to come.[9] Peptide-based drug discovery, therefore, deserves renewed attention.

The aforementioned revival has been fueled by a number of technological advances that have addressed the main limitation that has stymied the field for so long: the poor pharmacokinetic properties of peptides. Indeed, peptides generally exhibit poor oral bioavailability and high proteolytic susceptibility, requiring parenteral administration and exhibiting short half-lives.[1,3,10,11] Examples of the aforementioned palliative advances include *N*-methylation, cyclization to enable chameleonic behavior, stapling, the inclusion of D-amino acids, and peptoid macrocycles.[1,12−19] However, while these advances inform us about what to incorporate into the design of peptide screening libraries, they also highlight the limitations of current library-generation methods.

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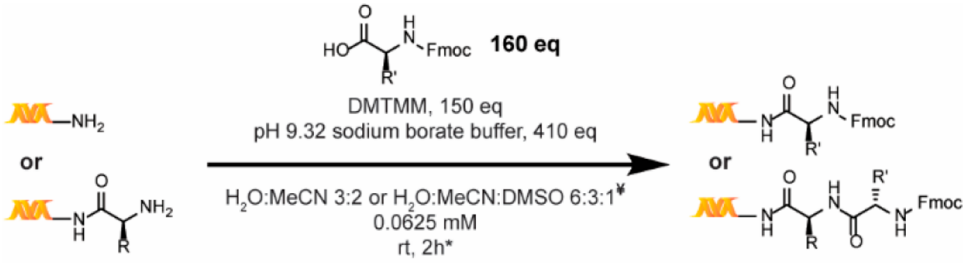
**Scheme 1. Developed General Amide Coupling Conditions for Single *Nα*-Fmoc-Amino Acid Building Blocks**[a]




[a]¥: Certain amino acids were not soluble in MeCN and had to be prepared in DMSO. *: Reactions were typically complete after 2 h but can be boosted for higher yields with the addition of 150 equiv of DMTMM at 2 h for reaction until 3 h; the chemical structure of the DNA tag (in yellow) is described in the Supporting Information.

Biological display methods—phage, ribosome, and mRNA display—are the protagonists of de novo peptide ligand discovery, but their main lacuna has been the inability to fully implement the lessons learned from pharmacokinetic optimization studies due to limited synthetic access to such analogs.[1] It is true, however, that the expansion of the genetic code to encode for noncanonical amino acids[20] and the engineering of flexible ribosomes[21] have significantly enhanced such access. An illustration is the $10^{14}$-member mRNA-display library produced by Szostak and co-workers[22] who were able to incorporate 12 unnatural amino acids into the design. Additionally, post translational modifications, such as cyclization and stapling, have been made possible in mRNA-display.[23] The chemical space accessible to biological display methods therefore promises to broaden as synthetic and recombinant methods converge.[1] However, such convergence is incomplete, which limits the chances of discovery and is incompatible with the pressing need for new peptide therapeutics.

DNA-encoded chemical libraries (DECLs) provide a potential solution. The value of DECLs in this context lies in the synergy of large numbers ($10^6$–$10^9$-member libraries)[24,25] and in their broader, ribosome-independent synthetic access. Theoretically, they could include any unnatural amino acid—in addition to all natural ones—as well as allow for a larger set of postsynthetic modifications thanks to a larger and expanding chemical toolkit.[26−35] An illustration of the advantage of DECLs—one of particular interest to us—lies in all-hydrocarbon stapled peptide drug discovery, whereby peptides are stapled into proteolytically stable, cell-permeable forms using the ring-closing metathesis reaction.[15] This area has borne the clinical candidate ALRN-6954, an all-hydrocarbon stapled α-helical peptide capable of inhibiting the p53-MDM2/MDMX PPIs in p53-dependent cancer therapy.[4] Such peptides require the incorporation of α,α-disubstituted unnatural amino acids, which are not currently accessible to biological display methods. Similarly, the ring-closing metathesis reaction remains unreported on the latter platform. In contrast, the latter reaction has been developed for DECLs,[35] while the incorporation of α,α-disubstituted amino acids is investigated and demonstrated in this work.

Hence, as a bridge to building peptide DECLs, we, herein, report (1) solution-phase, aqueous reaction conditions for the coupling of amino acid monomers to DNA and DNA-amino acid conjugates, (2) a screen of coupling efficiency between amino acid residues and DNA-amino acid conjugates to demonstrate the substrate tolerance of the developed conditions, (3) DNA-compatible protecting group strategies and the limitations thereof for relevant amino acids, (4) a strategy and reaction conditions for the coupling of α,α-disubstituted alkenyl amino acids as a tool for the generation of all-hydrocarbon stapled peptide DECLs, and (5) the synthesis of a fully deprotected DNA-decamer conjugate from amino acid monomers to illustrate the overall efficacy of the developed conditions.

## RESULTS AND DISCUSSION

Solid-phase peptide synthesis (SPPS) is one of the most established chemical processes owing to decades of development,[36−38] and the pleiotropy of protecting group strategies[39] and coupling reagents[40] available today enable the most synthetically challenging endeavors. Neri and co-workers[30] reported the relative efficiency of various amide coupling reagents for the conjugation of carboxylic acids to DNA. However, few of the substrates tested were amino acids, and the recommended amide coupling conditions—EDC/HOAt/DIPEA—proved inadequate, as explained below (see section on glutamine). Similarly, while amide coupling conditions have been reported for the generation of macrocyclic peptide and polyketide DECLs,[41,42] the amino acids/amino acid oligomers used were mostly hydrophobic, offering no significant protecting group strategies. Of note, DNA is sensitive to harsh conditions[43,44] (acidic and oxidative, for example), making Boc chemistry problematic and protecting group strategies limited.

**Developed Amide Coupling Reaction Conditions.** DECL builds are based on combinatorial chemistry, and high-yielding, substrate tolerant reaction conditions are desirable. Such conditions were developed, as summarized in Scheme 1.

The main challenge we faced was the limited solubility of amino acid/amino acid oligomers in aqueous media. Indeed, peptide insolubility is recognized as one of the most significant problems of peptide chemistry.[38] Moreover, since a protic medium is best for the suppression of nucleophilicity[45] and hence for the protection of the DNA tag from reagents,[44] solution-phase DECL reactions are ideally performed in cosolvent mixtures of high water proportion. A balance between amino acid/amino acid oligomer solubility and protection of DNA integrity was therefore empirically struck. Interestingly, most amino acids remained soluble in a buffered (pH ∼9.5) 40% acetonitrile (MeCN) aqueous solution. We hypothesized the ionization of the α-carboxyl group of the amino acid residues ($pK_a$ ∼ 2−3) enabled such solubility, which is not achievable if preactivation is used as a coupling strategy. Indeed, preactivated amino acids generally precipitated from the same medium and exhibited poor coupling


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**Table 1. Screen of Coupling Efficiency Between DNA-Amino Acid Conjugates and N$^{\alpha}$-Fmoc Amino Acids under the Conditions Summarized in Schemes 1, 2, and 4 (Boxed)[a]**

| | | % conv. | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Substrate # | Amino Acid —$NH_2$ | A | V | L | I | M | CHA* | P | F | Pg* | DPA* | W (Boc)$ | H (τ-(2-Ns)) | C (S-tBu)# | Q | D (OEpe) | Orn (NVOC)* | K (Boc) | S | T | Y |
| 1 | A | >99 | >99 | >99 | 81 | >99 | 87 | 97 | >99 | >99 | 92 | 78 | 52 | >99 | >99 | 93 | 81 | >99 | >99 | 92 | >99 |
| 2 | V | >99 | >99 | >99 | 91 | >99 | 74 | >99 | >99 | >99 | >99 | 93 | 42 | >99 | >99 | >99 | 84 | >99 | >99 | >99 | 90 |
| 3 | L | >99 | >99 | >99 | 89 | >99 | >99 | >99 | 95 | 94 | >99 | 89 | 49 | >99 | >99 | >99 | 84 | >99 | 91 | >99 | 95 |
| 4 | I | >99 | >99 | >99 | 92 | >99 | 86 | >99 | >99 | 93 | >99 | 74 | 47 | >99 | >99 | >99 | 85 | >99 | 92 | >99 | 95 |
| 5 | M | >99 | >99 | >99 | >99 | >99 | 84 | >99 | 92 | >99 | >99 | 81 | 38 | >99 | >99 | >99 | 92 | >99 | 93 | >99 | 88 |
| 6 | CHA* | >99 | >99 | >99 | >99 | >99 | 89 | >99 | >99 | >99 | >99 | >99 | 59 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 |
| 7 | P | >99 | >99 | >99 | >99 | >99 | 83 | 90 | >99 | 96 | >99 | 82 | 81 | >99 | >99 | >99 | 86 | >99 | 75 | 83 | 93 |
| 8 | F | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 33 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 91 |
| 9 | Pg* | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 46 | >99 | >99 | >99 | >99 | >99 | 87 | >99 | 84 |
| 10 | DPA* | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 14 | >99 | >99 | >99 | >99 | >99 | 80 | 94 | 54 |
| 11 | W(Boc) | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 45 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 |
| 12 | H△ | >99 | >99 | >99 | >99 | >99 | 88 | >99 | >99 | >99 | 75 | 73 | 46 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 70 |
| 13 | C(S-tBu) | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 82 | 30 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 83 |
| 14 | Q | >99 | 90 | >99 | >99 | >99 | 89 | >99 | >99 | 90 | 44 | 20 | 43 | 76 | >99 | >99 | 72 | 94 | 77 | 85 | 56 |
| 15 | D(Epe) | >99 | >99 | >99 | 86 | >99 | >99 | >99 | 97 | >99 | 95 | 96 | 40 | >99 | >99 | 95 | >99 | >99 | 38 | 59 | 72 |
| 16 | Orn(NVOC)* | 87 | >99 | >99 | 87 | 91 | 73 | >99 | >99 | 76 | 75 | >99 | 85 | >99 | >99 | >99 | 73 | >99 | 88 | >99 | 93 |
| 17 | K(Boc) | >99 | >99 | >99 | >99 | >99 | 82 | >99 | >99 | >99 | 86 | 59 | >99 | >99 | >99 | >99 | 87 | >99 | 95 | >99 | >99 |
| 18 | S | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 73 | 75 | >99 | 90 | >99 | >99 | 84 | >99 | 90 | >99 | 70 |
| 19 | T | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 84 | 85 | 77 | >99 | >99 | >99 | 89 | 81 | >99 | 87 | 85 | 78 |
| 20 | Y | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | >99 | 62 | T | >99 | >99 | 93 | >99 | >99 | >99 | >99 | 75 |

[a]All reactions were run with 2 nmol of DNA-amino acid conjugate for 2 h *: .CHA = cyclohexylalanine, Pg = phenylglycine, DPA = diphenylalanine, Orn = ornithine, S5 = α-methyl-α-pentenylalanine. #: Varying degrees of β-elimination were observed during coupling (see text for discussion). $: Up to 10% Boc removal during coupling. △: The 2-Ns protecting group fell off during DNA precipitation; % conv. = percent conversion calculated from the LC/MS signal, as described in the Supporting Information; **T** = see text for discussion (under tyrosine); green = ≥75% conversion; purple = 70−75% conversion; red = ≤60% conversion.

efficiency as a result. These observations led to a focus on the in situ activation of the amino acid building blocks in buffered aqueous media. While there exist useful studies on the subject,[46,47] the coupling reagent DMTMM showed remarkable substrate tolerance across the matrix of amino acid couplings studied. This is shown in Table 1. Most conversions are near-quantitative, which is desirable in a DECL build involving multiple steps. We note that other coupling reagents functioning via in situ activation may have similar substrate tolerance. The reported conditions showed such high efficiency, however, that further investigation was redundant. Also note that the DMTMM adduct (desired product mass +139 Da) that always forms during coupling is easily eliminated in the presence of piperidine during Fmoc removal (see Supporting Information).

**Amino Acids Requiring No Side-Chain Protection.** *Glutamine (Gln)—Fmoc-Gln-OH.* Protecting groups typically used for Gln—Trityl or 9-Xanthyl—are incompatible with this platform because of their need for acidic deprotection strategies.[39] However, Gln can be used unprotected and typically associated solubility issues were not encountered in this context. Moreover, the formation of pyroglutamine, a problem commonly encountered in acidic media, should be a nonissue on this platform. Unprotected Gln was therefore deemed safe to use in DECL builds. Indeed, as indicated in Table 1, its coupling efficiency is generally high across the matrix while no side reactions were observed (see Supporting Information). We note that because Gln has to remain unprotected, carbodiimide-based coupling reagents cannot be used due to their capacity to cause dehydration of primary amides to form nitriles.[39]

We also discovered that the substrate DNA-Gln-$NH_2$ degrades over time (onset ∼1 week) at −20 °C in neutral water to form a −17 Da byproduct (see Supporting Information). This mass change corresponds to the formation of the pyroglutamyl species. In contrast to the acidic conditions typically required for this side reaction, this occurs in neutral DNase/RNase-free neutral water. In addition, the −17 Da species does not couple to the next amino acid while the intact starting material does (see Supporting Information). While we have not investigated the identity of the side product, we hypothesize that the protic aqueous medium creates an


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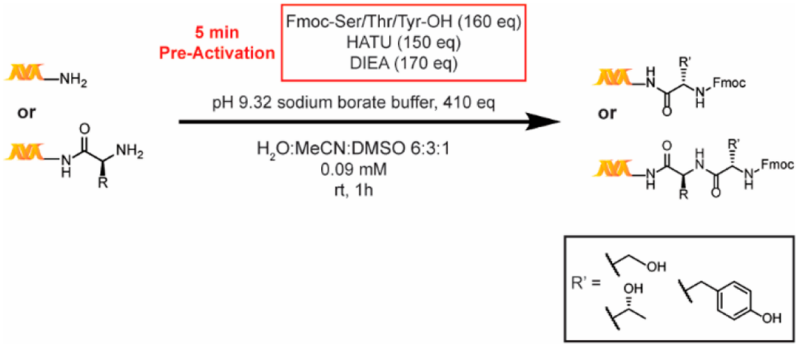
**Scheme 2. Developed Amide Coupling Conditions for the Coupling of Unprotected *Nα*-Fmoc-Ser, Thr, and Tyr[a]**




[a]Shaking the reaction is essential because of the significant precipitation upon mixing of the preactivation mixture to the aqueous portion of the reaction.

acidic enough environment for the pyroglutamyl species to form. We, thus, strongly recommend submitting the Gln residue to the next coupling reaction within a week from Fmoc removal while storing at −20 °C in neutral water.

*Methionine (Met).* Met is reported to undergo two side reactions in acidic media: oxidation to the sulfoxide and *S*-alkylation.[39] This is not a concern on this platform, and unprotected Met showed high coupling efficiency across the matrix studied (Table 1).

*Serine (Ser), Threonine (Thr), and Tyrosine (Tyr).* The most common protecting group strategies used for this class of amino acids include acid-labile ethers and carbonates.[39] While there currently exist no condition for the removal of ethers on DNA—even for the more labile phenolic ethers of Tyr—carbonates were found to hydrolyze in neutral water (data not provided).

Using this class of amino acids unprotected reportedly involves the risk of two side reactions: (1) *O*-acylation during coupling and (2) *O*−*N* migration during subsequent Fmoc removal.[39] However, none of these were observed under the conditions illustrated in Scheme 2. Note that these conditions are different from the general conditions summarized in Scheme 1, involving HATU and preactivation instead (see Supporting Information for a screen of coupling conditions). The unprotected alcohols generally showed high coupling efficiency across the matrix studied, as reported in Table 1. We, therefore, validate the use of unprotected Ser and Thr for the building of peptide DECLs.

*Tyrosine (Tyr).* While Tyr performed similarly to its aliphatic counterparts Ser and Thr (Table 1) under the conditions summarized in Scheme 2, its phenolic group exhibited undesirable reactivity, as categorized below.

*Extensive and Completely Reversible DMTMM-Capping.* When DNA-Tyr-$NH_2$ was subjected to the conditions described in Scheme 1, near-complete DMTMM-capping was observed. Such capping was easily reversed, however, in the presence of piperidine during the Fmoc removal procedure (see Supporting Information).

*Problematic Coupling to Fmoc-His(2-Ns)-OH.* Extensive capping of the phenolic oxygen with the 2-nitrobenzenesulfonyl protecting group of His was observed when DNA-Tyr-$NH_2$ was coupled with Fmoc-His(2-Ns)-OH under the optimized conditions (HATU (150 equiv), DIPEA (170 equiv), pH 3.32 sodium borate buffer (410 equiv), $H_2O$:MeCN:DMSO 6:3:1, 0.09 mM, rt, 1 h). In addition, the coupling efficiency was low (54%) and an unidentified byproduct was formed. While capping was largely reversed during a second coupling round, the unidentified byproduct persisted (+227 Da), thus hindering the yield of the reaction (see Supporting Information). Note that this would not occur if Tyr were incorporated after His in a DECL build since the 2-Ns group falls off soon after the incorporation of His.

While these problematic instances do not disqualify unprotected Tyr, they do highlight the reactivity of the phenolic moiety and we caution against its indiscriminate use in peptide DECL builds. Unable to provide a protecting group at this time, our recommendations for Tyr are as follows: (1) A build with both His(2-Ns) and Tyr is only safe if His(2-Ns) is incorporated before Tyr, and (2) all other amino acids tested in this work are compatible with unprotected Tyr, so that peptide DECL designs should be able to incorporate it flexibly enough.

**Developed Protecting Group Strategies.** Unlike biological display methods, synthetic endeavors such as DECLs require protecting group strategies for the suppression of side-chain reactivity. However, member peptides would need to be fully deprotected after DECL synthesis for selection experiments to be successful. Because DNA is sensitive to acidic conditions (pH > 4 is our benchmark), traditional protecting groups requiring highly acidic deprotection conditions[39] cannot be used. Adapted strategies thus had to be developed, as are discussed below.

*Aspartic Acid (Asp)—Fmoc-Asp(OEpe)-OH.* The typical side-chain protecting group used for Asp in Fmoc chemistry is the *tert*-butyl ester.[39] However, Asp is prone to piperidine-induced aspartimide (Asi) formation during Fmoc removal,[48] leading to deprotection of the *β*-carboxyl group, racemization at the *α* carbon, and to the formation of the *β*-peptide as the major product upon ring opening.[49] In a stapled peptide DECL, such rearrangement would significantly change the structure of the putative library members and would render screening results difficult to interpret. The minimization of the Asi pathway is therefore crucial.

While the aspartimide species was never observed during Fmoc removal, the elimination of the *tert*-butyl ester protecting group was (see Supporting Information), which was hypothesized to originate in the Asi problem. The strategy used to counter this was a combination of acid-[50] and steric bulk-mediated[51] minimization of Asi formation. This is illustrated in Table 2. Adding formic acid (5%) to the 20% piperidine Fmoc removal mixture limits the deprotonation of the peptide backbone nitrogen preceding the Asp residue—


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**Table 2. Minimization of the Aspartimide Formation and Protecting Group Strategy for the Aspartic Acid Building Block[a]**


| R | X | t = 15 min | 45 min |
|---|---|---|---|
| t-Bu = | 10% piperidine | 27 (73) | - |
| Epe = | 10% piperidine | 83 (17) | - |
| | 20% piperidine 5% formic acid | - | 96 (4) |

% desired product at time t (% Fmoc + R removal at time t)

[a]It consistently takes ∼45 min for complete Fmoc removal under the recommended 20% piperidine/5% formic acid condition; the numbers in the table are percent conversions determined via LC/MS, as described in the Supporting Information; the syntheses of the substrates are described in the Supporting Information.

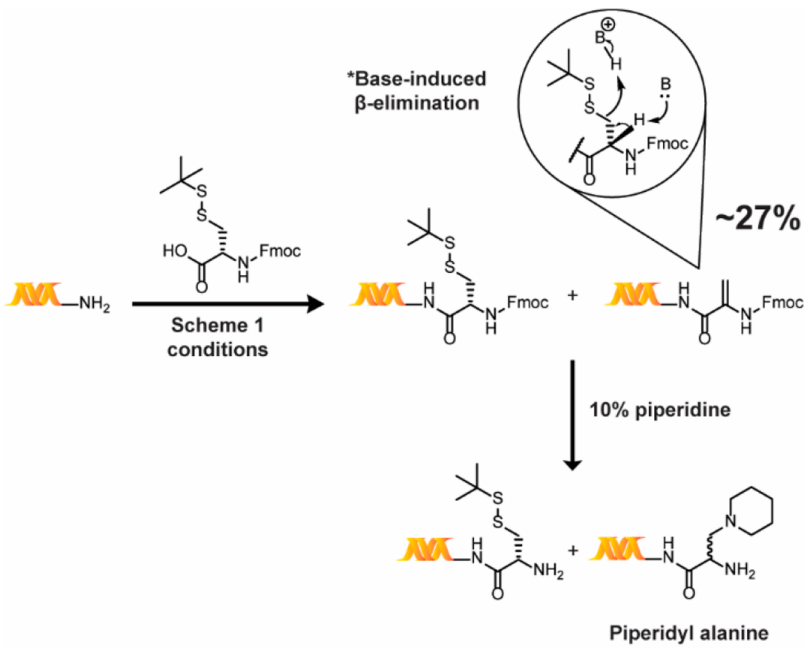
note that it also significantly reduces the rate of Fmoc removal (45 vs 20 min). The increased steric bulk offered by the 3-ethylpentyl (Epe) ester hinders the formation of the five-membered ring between the aforementioned backbone nitrogen and the β-carbonyl carbon. Note that, while *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone protection completely suppresses aspartimide formation,[52] it would also affect hydrogen-bonding and folding of the peptide, which would be undesirable in a screening library. Also note that the Asi formation is only minimized by the reported conditions and not suppressed. The accumulative formation of Asi is, therefore, to be expected. The higher the number of coupling steps and the earlier the incorporation of the Asp residue, the larger the extent of Asi-related disruption of the intended peptide structure. The proof-of-concept DNA-decamer conjugate built in this work (see below) includes an Asp residue early on and provides an idea of what to expect should Asp be a part of a peptide DECL. Finally, while 10% piperidine can be used for Fmoc removal, once Asp(OEpe) has been added to the peptide chain, every subsequent deprotection step has to be carried out using 20% piperidine/5% formic acid (see Supporting Information for preparation guidelines).

As for the removal of the Epe protecting group, base-catalyzed ester hydrolysis (pH 9.5 sodium borate buffer, 80 °C, 48 h)[26] proved adequate. The application of this protecting group strategy is demonstrated in the synthesis of the DNA-decamer conjugate (see below). Of note, it is possible that Asi occurs during this basic deprotection step. However, given the high temperature and consequent conformational freedom, and the steric advantage of water as a nucleophile, we believe hydrolysis is the major deprotection pathway. Given the limited analytical methods available to this platform, however, this remains conjectural and would need to be validated with further studies.

*Cysteine (Cys)—Fmoc-Cys(StBu)-OH.* The protection of Cys is paramount in peptide synthesis due to the nucleophilicity of the thiol side chain and to the formation of intra- and intermolecular disulfides in air.[39] While traditional protecting groups (Acm or Trt) are maladapted to this platform, the −StBu group was the only one we found to be satisfactory. Its coupling efficiency is illustrated in Table 1.

There was one major caveat, however. A −122 Da byproduct forms during the coupling of Cys(StBu), which corresponds to base-catalyzed β-elimination[39] (∼27% when coupled to the DNA headpiece and to varying extents, <27%,

**Scheme 3. Proposed Mechanism for the Side Reactions Observed during the Coupling of *Nα*-Fmoc-Cys(StBu) to DNA and DNA-Peptide Conjugates[a]**




[a]*: It is unclear when this base-induced β-elimination happens. It could be before (on the activated acid) or after coupling to the DNA headpiece or during both stages.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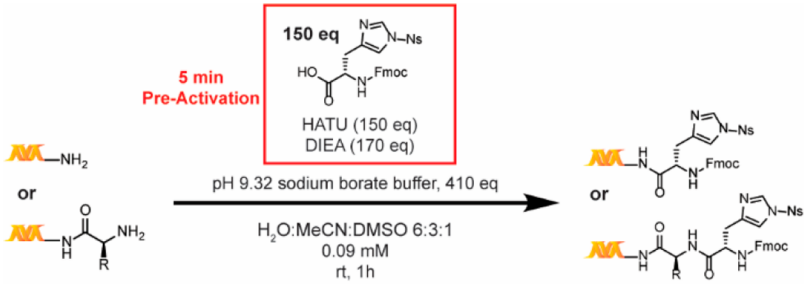
**Scheme 4. Developed Amide Coupling Conditions for the Coupling of *Nα*-Fmoc-His(2-Ns)-OH[a]**




[a]Shaking the reaction is essential due to significant precipitation upon mixing of the preactivation mixture to the aqueous portion of the reaction.

when coupled to DNA-amino acid conjugates; see Supporting Information). Additionally, the byproduct shifts in mass by +85 Da during Fmoc removal, which corresponds to the formation of the piperidyl alanine species[39] (see Supporting Information). These side reactions are illustrated in Scheme 3. Furthermore, both putative products formed after Fmoc removal are capable of coupling to the next amino acid, which indeed happens (see Supporting Information). Every Cys residue added to the peptide chain can therefore lead to the incorporation of unwanted racemized piperidyl alanine-bearing residues and potentially make the interpretation of screening results difficult. However, no further deprotection is observed upon coupling of the next amino acid (see Supporting Information). Given the majority of the material (~73%) is potent and that only $10^5$ copies[53] of the binder are required during screening, the incorporation of Cys could be considered should a DECL build require it.

Reductive deprotection conditions were necessary for this strategy. Incubating DNA-Cys(StBu)-$NH_2$ in HEPES 1× ligation buffer (30 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM adenosine triphosphate, pH 7.8) for 48 h resulted in the complete removal of the −StBu protecting group (see Supporting Information).

There is always the possibility of disulfide formation when unprotected Cys is present.[39] While the aforementioned mild deprotection conditions are convenient, they also imply that codon ligation—typically performed in HEPES 1×—should be done without dithiothreitol (DTT), which is possible. Finally, selection experiments are routinely performed under reducing conditions, which should avoid any disulfide-related issues a fully deprotected, Cys-containing peptide DECL could encounter.

We, therefore, propose −StBu as a protecting group for Cys but advise caution when incorporating it into a build. It can be included, but the yield of the library will suffer.

*Histidine (His)—Fmoc-His(2-Ns)-OH.* His presents two main issues: (1) two nucleophilic centers, the $N^{\pi}$ and $N^{\tau}$ imidazole nitrogens, and (2) $N^{\pi}$-mediated racemization of the activated amino acid.[39] Protection of either nitrogens with an electron-withdrawing group has been shown to minimize both issues.[54,55] The most oft used protecting group in Fmoc-based SPPS is Trt.[39] However, this cannot be used here for a lack of DNA-compatible deprotection conditions. Striking a balance between the need for an electron-withdrawing protecting group and a DNA-compatible deprotection strategy, we synthesized and validated $N^{\alpha}$-Fmoc-$N^{\tau}$-(2-nitrobenzylsulfonyl)-L-His-OH for this application (see Supporting Information).

The use of His(2-Ns) required the adoption of alternative coupling conditions (see Supporting Information for coupling reagent screen), as illustrated in Scheme 4 (same conditions as in Scheme 2). Given the relatively low coupling efficiency (Table 1), however, we recommend double coupling (couple, precipitate, couple) to achieve satisfactory conversion.

Finally, it was found that the Ns protecting group spontaneously falls off, but only after coupling to the amine (see Supporting Information). While thiolysis is the known method for nosyl removal on DNA,[56] this facile hydrolysis proved convenient. Although this exposes the nucleophilic imidazole nitrogens, we did not observe any side reactions during peptide elongation nor during NVOC removal (see Supporting Information).

*Lysine (Lys)—Fmoc-Lys(Boc)-OH.* The traditional Boc protecting group strategy proved adequate in this context. As indicated in Table 1, the $N^{\alpha}$-Fmoc-$N^{\varepsilon}$-Boc-L-Lys residue couples efficiently across the matrix studied. Robust, DNA-compatible Boc removal conditions have been developed[43] and their adaptation (pH 9.5 sodium borate buffer, 80 °C, 48 h) is demonstrated during the deprotection of the DNA-decamer conjugate (see below).

*Tryptophan (Trp)—Fmoc-Trp(Boc)-OH.* Trp is often used unprotected in Fmoc-based peptide synthesis, although it can react with deprotection byproducts of Mtr, Pmc, or Pbf groups[39]—none of which are used here. However, because unprotected Trp exhibited solubility issues under the developed conditions, and as an added precaution, we used a protected version. Similar to Lys, the $N^{in}$-Boc protecting group strategy proved adequate. As reported in Table 1, the coupling efficiency was relatively lower overall, and boosting with more DMTMM at the 2 h time point may be required to achieve better conversion during a library build. Interestingly, the Boc group falls off to some extent (up to 10%) during coupling, more significantly (additional 20%) during Fmoc removal, and further over time (~1 month, additional 40%) in water at −20 °C (see Supporting Information). Indeed, this exposes the indole nitrogen and nullifies the attempted protection. However, no side reactions were observed during peptide elongation, nor during NVOC removal (see Supporting Information).

The deprotection conditions are the same as the ones used for Lys (see Supporting Information).

**Problematic Amino Acids and Developed Solutions.** *Arginine (Arg)—Orn(NVOC) to Arg(Boc)$_2$.* Side-chain protection of Arg remains a challenge in peptide synthesis because of the reactivity of all three nitrogens of the guanidine moiety.[39] Masking two of them with bulky electron-withdrawing groups minimizes those side reactions, which was initially pursued as a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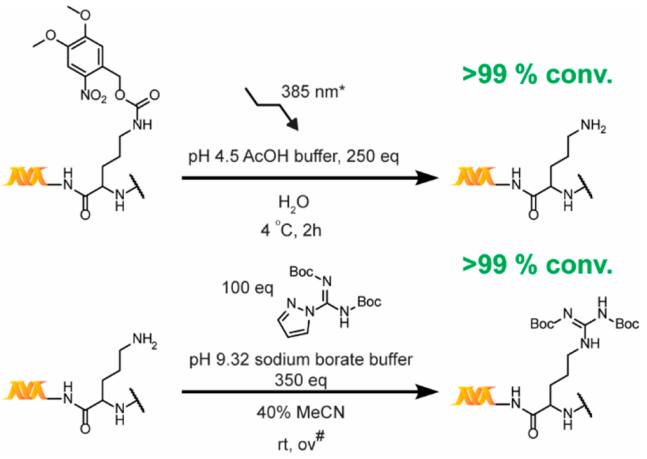
strategy. The only commercially available DNA-compatible protected Arg is $N^{\alpha}$-Fmoc-$N^{\omega}$,$N^{\omega}$-$(Boc)_2$-L-Arg. However, this amino acid does not couple efficiently (see Supporting Information for coupling reagent screen) and a work-around had to be developed, as is illustrated in Scheme 5.

**Scheme 5. In Situ Generation of $R(Boc)_2$ from Orn(NVOC)[a]**




[a]*: A UV lamp was used to generate the necessary irradiation (see Supporting Information for details about lamp used); the Boc protecting groups can be removed using the known Boc removal conditions (pH 9.5 sodium borate buffer, 80 °C, 48h) (see Supporting Information). #: These conditions were adapted from Dawadi, S. (manuscript in preparation).

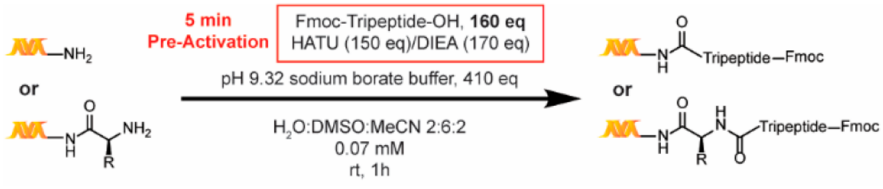
The in situ conversion of ornithine (Orn) to Arg not only solves the issue of coupling efficiency but also addresses that of side chain reactivity since Arg can be generated at the end of a peptide build. Moreover, the NVOC group[57] offers orthogonal protection, allowing for the preservation of the primary amine of Boc-protected Lys, while Arg is generated. The compatibility of this strategy with the unprotected indole nitrogen of Trp and the imidazole nitrogens of His was also validated (see Supporting Information). As shown in Table 1, Fmoc-Orn(NVOC)-OH couples efficiently across the matrix studied.

As for the deprotection of the generated $Arg(Boc)_2$, the general *N*-Boc removal conditions (pH 9.5 sodium borate buffer, 80 °C, 48 h) proved adequate (see Supporting Information).

*Nα-Fmoc-2-(4′-pentenyl)-L-alanine (S5)—Tripeptide Sandwiching.* All-hydrocarbon stapled peptides use α,α-disubstituted alkenyl amino acids for the production of [*i*, *i* + 3]/[*i*, *i* + 4]/[*i*, *i* + 7] peptides via the ring-closing metathesis reaction.[15] However, because of their quaternary alpha center, those amino acids are notoriously difficult to couple, and often require double coupling in SPPS.[58] Such difficulty translated to this platform and S5 showed unsatisfactory coupling efficiency (<20%) under all conditions tested (see Supporting Information). An alternative route therefore had to be developed in the form of a tripeptide sandwich of S5, which exploits the higher coupling efficiency of the flanking amino acids. The effectiveness of this technique is illustrated in Table 3.

Unlike amino acid monomers, tripeptides were not soluble under the conditions described in Scheme 1. Instead, a high percentage of organic cosolvent (80%) was required to ensure reaction homogeneity. As discussed earlier, this is not ideal and DNA-compatible organic cosolvents[56] had to be carefully selected. DMSO was chosen for its high miscibility with water, its high solvation capacity toward the tripeptides used and its validation as a safe cosolvent during a number of our past and current projects. The presence of MeCN—routinely used in DNA-encoded chemistry—also proved necessary to ensure reaction homogeneity. A corollary of this largely organic solvent system is the access to preactivation as a coupling strategy. Indeed, preactivation with DEPBT/DIEA or HATU/DIEA caused no precipitate upon mixing with the aqueous portion of the reaction, and exhibited higher coupling efficiency than DMTMM (see Supporting Information). Another corollary is the enhanced exposure of the *N*-terminal

**Table 3. Tripeptide Sandwich Strategy for the Coupling of α,α-Disubstituted Alkenyl Amino Acids to DNA/DNA-Amino Acid Conjugates[a]**




| Substrate # | | | | |
|---|---|---|---|---|
| 21 | | 98 | >99 | >99 |
| 22 | | 95 | >99 | >99 |

[a]The preactivation mixture was in DMSO only; the tripeptides were made on solid phase (see Supporting Information); all reactions were run with 2 nmol of DNA/DNA-amino acid conjugate; the numbers in the table represent percent conversions determined via LC/MS, as described in the Supporting Information.



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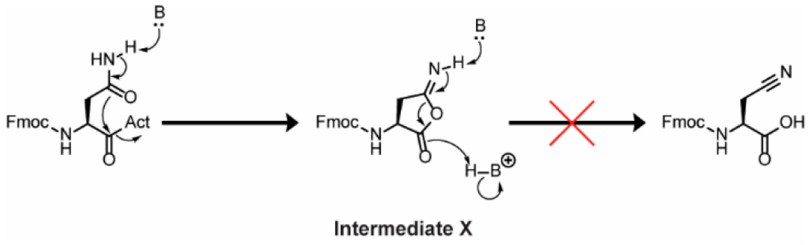
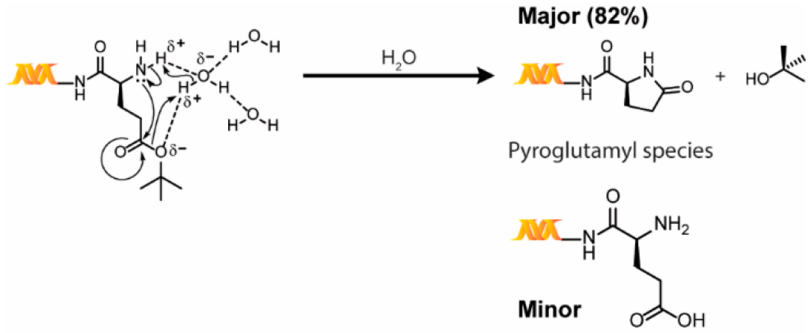
**Scheme 6. Dehydration of Activated Asn and Trapping as Intermediate X as the Proposed Explanation for the Unsuccessful Coupling to DNA/DNA-Amino Acid Conjugates**[a]




[a]Act = activating group incorporated through reaction with coupling reagent; Intermediate X cannot couple to a free amine.

**Scheme 7. Proposed Mechanism for the Degradation of DNA-Glu(OtBu)-NH2 in Neutral Water**[a]




[a]This side reaction occurs in DNase/RNase-free water at −20 °C once the Fmoc group in DNA-Glu(OtBu)-Fmoc has been removed; we propose that the hydrogen-bonding network provided by the aqueous medium facilitates cyclization and the consequent elimination of the *tert*-butyl protecting group.

of the DNA-peptide conjugate to capping by the coupling regent. While DEPBT and DMTMM caused extensive capping, HATU did not and significantly outperformed the other coupling regents tested (see Supporting Information). We, therefore, recommend HATU as the coupling reagent of choice for the coupling of tripeptide sandwiches of S5.

Of note, the developed tripeptide coupling conditions could be very useful beyond the incorporation of S5. DECL builds typically consist of 3−4 cycles, and using tripeptides would minimize the number of cycles necessary to reach the desired peptide length. The building of a 12-mer, for example, would require only 4 tripeptide- vs 12 single-amino acid coupling steps.

**Unsolved Cases.** *Asparagine (Asn).* Like Gln, Asn is often used unprotected, although the dehydration of its amide side chain to a nitrile is a risk in Fmoc chemistry.[39] While no solubility issues—another problem associated with unprotected Asn—were observed, the coupling of Asn failed under all conditions tested. In contrast, the coupling of $N_{\alpha}$-Fmoc-L-Asn(Trt)-OH to the DNA headpiece under the conditions reported in Scheme 1 was relatively successful (see Supporting Information). We hypothesize that the activated Asn species remains trapped as intermediate X (Scheme 6) during dehydration, unable to couple to the free amine of the DNA headpiece or to that of a DNA-amino acid conjugate.

Traditional protecting groups employed for Asn—trityl or 9-xanthyl—require strongly acidic deprotection conditions and are incompatible with this platform. Unfortunately, no DNA-compatible alternative has been found yet, precluding us from offering a protecting group strategy at this time.

*Glutamic Acid (Glu).* Thanks to its longer three-carbon—relative to Asp's two-carbon—side chain, Glu is not known to form the analogous "glutimide" species. However, it is known to form the pyroglutamyl species—5-membered ring analogous to the Asi species—in the presence of HF.[59] Despite the absence of such harsh conditions in the present context, when DNA-Glu(OtBu)-Fmoc is subjected to 10% piperidine, the major product has a mass of ∼74 Da lower than that of the desired product. This corresponds to the loss of a *tert*-butyl group—∼56 Da—and of a $H_2O$ molecule—∼18 Da. In fact, the transformation continues beyond Fmoc removal in neutral water at −20 °C (∼1 week) until only the −74 Da (*tert*-butyl deprotection and dehydration, 82%) and the −56 Da (*tert*-butyl deprotection, 18%) byproducts are left (see Supporting Information). The continued degradation of the material in the absence of piperidine suggests that the mechanism involved is different from that of Asi formation and is independent of piperidine. When this degraded material was subjected to a coupling reaction with $N^{\alpha}$-Fmoc-L-alanine, the −74 Da byproduct did not react, while the −56 Da one did (see Supporting Information). This suggests that the N-terminal has been made unavailable in the −74 Da species. We hypothesize that the protic aqueous medium allows for the deprotected *N*-terminal to cyclize onto the γ-carbonyl carbon, ejecting *tert*-butanol and forming the unreactive pyroglutamyl species. A proposed mechanism is illustrated in Scheme 7.

Similar to the technique employed in the case of Asp, steric bulk of the γ-ester may limit such degradation. Unfortunately the synthesis of Fmoc-Glu(OEpe)-OH remains intractable in our hands, and no protecting group strategy for Glu is offered at this time.

**On-DNA Synthesis of Peptide Decamer.** To illustrate the overall efficacy of the conditions summarized in Scheme 1, substrate **23** shown in Scheme 8 was synthesized through the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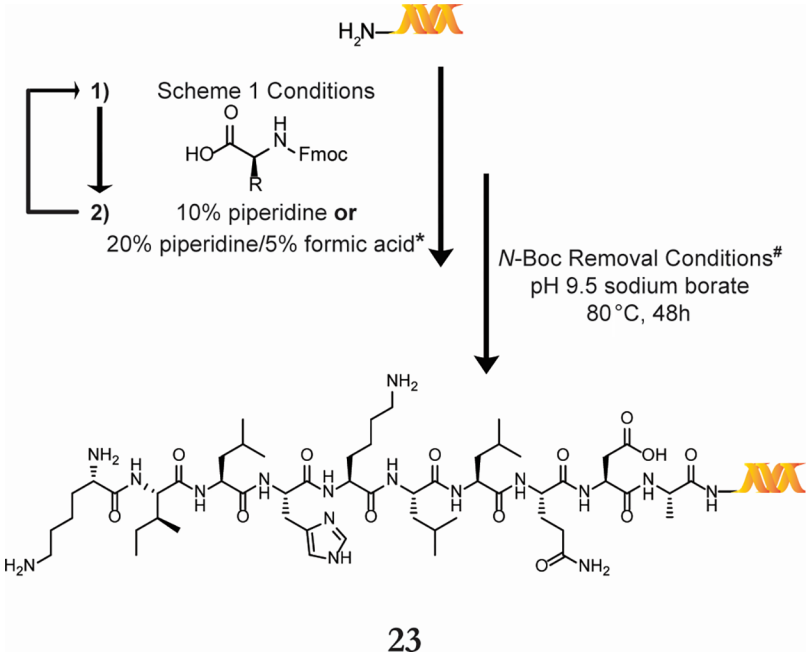
**Scheme 8. Synthesis of Fully Deprotected DNA-Decamer Conjugate**[a]




23

[a]*: After the incorporation of − Asp(OEpe)-, Fmoc removal was performed in the presence of formic acid. #: Only the Boc and Epe protecting groups were left at the end of the synthesis, the 2-Ns group of His having spontaneously fallen off; the side-chain protecting groups used for relevant Fmoc-amino acid building blocks were as discussed in the text; the synthesis is detailed in the Supporting Information.

successive coupling and *N*-terminal deprotection of its constituent amino acid building blocks, and through the application of the developed protecting group strategies (see Supporting Information for step-by-step synthesis and characterization). Of note, before the subjection of the peptide to the *N*-Boc removal conditions—which also removes the Epe group—at the end of the synthesis, a 20% Epe removal level was recorded (see Supporting Information). As mentioned earlier, Asi formation and the consequent elimination of the aspartic acid side chain protecting group, Epe, is only minimized by the developed strategy. Given the early incorporation of Asp into the sequence and the relatively large number of Fmoc removal steps (i.e., 10) we deem the developed Asi minimization strategy effective. Admittedly, however, a 20% Asi-mediated Epe elimination level is significant. Moreover, as also mentioned above, it is unknown whether the removal of the Epe group under the basic *N*-Boc removal conditions results in further Asi formation. This would need further investigation. Aside from the Asi issue however, the synthesis of **23** highlights the efficacy of the developed conditions (Scheme 1) for building peptides on DNA.

The amino acid sequence of **23** is based on the nuclear receptor box (NR-box) of steroid receptor coactivators (SRCs) of the estrogen receptor ER$\alpha$.[60] This PPI is a target of interest to our laboratory and the successful synthesis of **23**—caveats aside—was a promising prelude to future peptide DECL productions.

## CONCLUSION

In conclusion, we have (1) developed substrate tolerant conditions for the coupling of Fmoc-amino acid monomers to DNA and DNA-peptide conjugates, (2) developed DNA-compatible protecting group strategies for relevant amino acids while highlighting pitfalls and unsolved cases, as an opportunity for further research, (3) developed a tripeptide sandwich strategy for the coupling of $\alpha,\alpha$-disubstituted alkenyl amino acids as a bridge to the DECL-enabled exploration of stapled peptide chemical space, and (4) demonstrated the potency of the developed monomer-coupling conditions through the synthesis of a fully deprotected DNA-decamer conjugate. This work should provide a roadmap for the addition of DECLs to the arsenal available to peptide-based drug discovery, which is currently dominated by synthetically restricted, ribosome-dependent biological display methods.

## EXPERIMENTAL DETAILS

While DMTMM-capping of the DNA-chemical conjugate is rampant and deleterious to codon ligation (we believe it reacts with the 5′-Phos of the DNA headpiece), piperidine-enabled Fmoc removal completely reverses it (see Supporting Information; heating during Boc removal is also known to reverse such capping). Also, the presence of the Fmoc group affects the quality of the MS signal and can make it difficult to determine the efficiency of the coupling reaction. In such cases, we recommend using the disappearance of the starting material as confirmation of complete conversion until the Fmoc group is removed and the quality of the MS signal improves drastically (see Supporting Information).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.0c00144.

Reaction conditions, NMR spectral data for all new compounds, LC/MS of on-DNA products, and chiral analytical data (PDF)



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### Author Contributions

O.B.C.M. designed and executed the entire investigation. N.S. and S.C. provided guidance and training throughout the investigation. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.


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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

DEPBT, 3-(diethoxy-phosphoryloxy)-1,2,3-benzo[*d*]triazin-4(3*H*)-one; DIPEA, *N*,*N*′-diisopropylethylamine; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; EDC, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimides; HATU, 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate; HOAT, 1-hydroxy-7-azabenzotriazole.

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