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Communication

On-DNA Morita-Baylis-Hillman Reaction: Accessing Targeted Covalent Inhibitor Motifs in DNA-Encoded Libraries

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ABSTRACT: We herein present the first application of the on-DNA Morita-Baylis-Hillman (MBH) reaction for the creation of pharmaceutically relevant targeted covalent inhibitors (TCIs) with an α-hydroxyl Michael acceptor motif. Adapting a DNA-compatible organocatalytic process, this MBH reaction for covalent selection-capable DNA encoded library (DEL) synthesis grants access to densely functionalized and versatile precursors to explore novel chemical space for molecule recognition in drug discovery. Most importantly, this methodology sheds light on potentially unexpected reaction outcomes of the MBH reaction.

For the past two decades, DNA encoded library technology (DELT)[1] has been continuously evolving and benefited from the multidimensional advancements of new on-DNA chemical reactions, improved selection methods, increasingly affordable nucleotide sequencing, and ameliorated data analysis approach to reveal the enriched binders.[2,3] Many academic groups and industrials have turned their attention on the field and have brought significant technological advances which have made DEL screening a viable alternative to high-throughput screening (HTS) for early drug discovery campaigns.[4,5] Compounds discovered from DEL selection studies have now progressed to clinical trials.[6,7] DNA encoded libraries (DELs) of superior quality are driven by innovative DEL design,[8−11] diverse chemical space,[12,13] improved druggable profiles,[14] and efficient chemical transformation, and not by any astronomical numbers of on-DNA compounds created by a small panel of reactions.[15] Thus, the implementation of new DNA-compatible reactions has stimulated the exploration of yet uncharted chemical spaces via the expansion of the structural diversity of DELs.[16−18] However, there are still a limited number of DEL synthesis-competent reactions, particularly those showcasing a vast substrate scope, reduced number of potential byproducts, and high conversion.

To expand the diversity of the DEL-amenable chemical toolbox, especially in the homologation section, methods were sought to forge C−C bonds through a variety of coupling including Suzuki coupling,[19] olefin metathesis,[20] aldol condensation,[21] cycloaddition,[22] and radical-based cross-coupling.[23] Nonetheless, considering the constant need for C−C bond formation in library builds, the lack of on-DNA access to certain key pharmaceutically relevant motifs, and our thirst for the development of unique and chemical space enriching methodologis,[24−29] we naturally took interest in this field and approached it through a new lens.

Targeted covalent inhibitors (TCIs) or targeted covalent drugs are rationally designed inhibitors that can bind irreversibly to their respective target proteins.[30,31] Over the past century, the pharmaceutical industry has developed several effective covalent drugs that have greatly impacted human health. Penicillin, omeprazole, clopidogrel, and aspirin, discovered by serendipity in phenotypic screens,[32,33] were only the stammering of this fascinating drugs class and efforts are now targeted at the use of rational drug design to generate extremely selective covalent inhibitors known as targeted covalent inhibitors.[34] Examples such as Paxlovid, indicated treatment for SARS-CoV-2,[35] Ibrutinib, an oral Bruton's tyrosine kinase inhibitor, Osimertinib,[36] an irreversible epidermal growth factor receptor (EGFR) inhibitor,[37] and others are particularly explicit (Figure 1).[38] As a general trend, targeted covalent drugs feature a Michael acceptor as the core binding motif to the targeted POI. However, introduction of such motif to equip on-DNA compounds is mostly limited to the acylation of a free amine with acrylic acid and Heck

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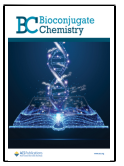

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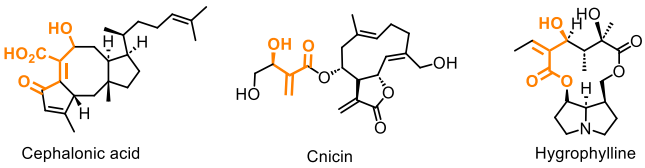


Figure 1. Targeted covalent acting drugs and natural products with a α-hydroxyl Michael acceptor motif.

reaction,[39,40] or alike. Thus, we saw here room for improvement, which prompted us to tackle the synthesis of on-DNA molecules functionalized with warheads such as Michael acceptors, through a more versatile and less substrate-constraining approach.

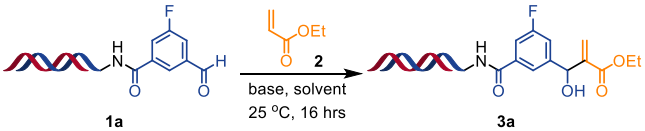
The Morita-Baylis-Hillman (MBH) reaction, and its variants, is one of the most useful carbon−carbon bond-forming transformation. Involving readily available starting materials, it lies in the coupling of the α-position of an activated alkene and the sp^2 hybridized electrophilic carbon center such as an aldehyde, ketone, ester, or imine, to name only a few. Typically requiring an organocatalytic nucleophilic catalyst such as tertiary amines or phosphines,[41] this simple process produces densely functionalized molecules with newly added chirality under metal-free conditions. DABCO is one of the most utilized tertiary amine catalysts for this reaction.[42] Additionally, the formed products are versatile synthons and allow for various subsequent postfunctionalization and the reaction has naturally become a formidable synthetic instrument.[42] Yet, some impediments refrain from employing this transformation routinely. It does, in fact, frequently struggle with slow rates, prolonged reaction times, and poor yields.[43] Nonetheless, despite the known difficulties of the MBH reaction, an on-DNA version of this transformation should be surveyed for the synthesis of a novel class of covalent capable DELs. Thus, we embarked on the exploration of this exciting reactivity in the hopes that a sagacious choice of reaction conditions could circumvent these inherent synthetic challenges.

Our study kicked off with an investigation on the reaction conditions using 3-fluoro-5-formyl-*N*-benzamide DNA conjugate **1a** and ethyl acrylate **2**. To optimize the reaction conditions, a range of variables, including the concentration of reactants, different bases or buffers, along with reaction temperature, and solvent mixtures and their ratios were assessed.

Our initial assessment revealed the necessity of the presence of both a base and promoter was revealed (Table 1, entries 1−4). Although the reaction conversion was quite low (19%) when 1,4-diazabicyclo[2.2.2]octane (DABCO) was utilized as base and promoter (entry 2), the results showed promises. Comparing the MBH adduct with a prepared DNA conjugate derived from an off-DNA authentic sample revealed that they were identical (see SI9.19). Indeed, it proved that the MBH reaction worked in water under mild conditions, in contrast with previously described conditions employing high heating,[44,45] micellar media,[46] fluorinated cosolvents,[47] or with ionic liquids.[48] Next, the use of DABCO was assessed and illustrated the necessity of a large excess amount of base, as 1000 equiv (333.3 mM) was necessary for the highest conversion (53%, entry 7).

Sequentially, the solvent effect was examined and illustrated that acetonitrile/water (2:1) was superior to other mixed solvents (entries 10−13). The ratio of acetonitrile/water was

Table 1. Reaction Optimization of Baylis-Hillman Reaction of Ethyl Acrylate with DNA-Conjugate 1a[a]



Entry	Base	Base(eq./mM[b])	2(eq./mM[b])	Solvent	3a (%)[c]
1	TMG	200/66.6	200/66.6	$CH_3CN : H_2O = 2 : 1$	0
2	DABCO	200/66.6	200/66.6	$CH_3CN : H_2O = 2 : 1$	19
3	$NaHCO_3$	200/66.6	200/66.6	$CH_3CN : H_2O = 2 : 1$	0
4	Cs_2CO_3	200/66.6	200/66.6	$CH_3CN : H_2O = 2 : 1$	0
5	DABCO	100/33.3	200/66.6	$CH_3CN : H_2O = 2 : 1$	0
6	DABCO	500/166.6	200/66.6	$CH_3CN : H_2O = 2 : 1$	41
7	DABCO	1000/333.3	200/66.6	$CH_3CN : H_2O = 2 : 1$	53
8	DABCO	1000/333.3	100/33.3	$CH_3CN : H_2O = 2 : 1$	48
9	DABCO	1000/333.3	500/166.6	$CH_3CN : H_2O = 2 : 1$	70
10	**DABCO**	**1000/333.3**	**1000/333.3**	**$CH_3CN : H_2O = 2 : 1$**	**79**
11	DABCO	1000/333.3	1000/333.3	$THF : H_2O = 2 : 1$	41
12	DABCO	1000/333.3	1000/333.3	$DMF : H_2O = 2 : 1$	37
13	DABCO	1000/333.3	1000/333.3	$EtOH : H_2O = 2 : 1$	58
14	DABCO	1000/333.3	1000/333.3	$CH_3CN : H_2O = 1 : 1$	71
15	DABCO	1000/333.3	1000/333.3	$CH_3CN : H_2O = 1 : 2$	25
16[d]	DABCO	500/833.3	200/333.3	$CH_3CN : H_2O = 2 : 1$	92
17[d]	**DABCO**	**200/333.3**	**200/333.3**	**$CH_3CN : H_2O = 2 : 1$**	**92**
18[d]	DABCO	100/166.6	100/166.6	$CH_3CN : H_2O = 2 : 1$	63
19[d]	DABCO	50/83.3	100/166.6	$CH_3CN : H_2O = 2 : 1$	51
20[d]	DABCO	50/83.3	50/83.3	$CH_3CN : H_2O = 2 : 1$	47

[a]**1a** (10.0 nmol in H_2O, 1.0 equiv, 10.0 μL), **2** (10.0 μL), base (10.0 μL), solvent, total volume is 30.0 μL, 25 °C, 16 h. [b]Final concentration. [c]Conversions were determined by LC-MS. [d]**1a** (10.0 nmol in H_2O, 1.0 equiv, 2.0 μL), **2** (2.0 μL), base (2.0 μL), solvent, total volume is 6.0 μL, 25 °C, 16 h.

then adjusted. Higher water content reduces the rate of conversion, presumably making reactant **2** less soluble (entries 14−15). Interestingly, although seminal attempts to limit DABCO usage resulted in a drop in conversion, the MBH reaction converted most effectively with 200 equiv (333.3 mM) of DABCO when **1a** concentration was increased 5-fold (entries 16 to 20). The enhanced conversion was presumably due to the higher concentration of reagents (see SI 5.4 for details).

Remarkably, the same conditions led to the highest conversion upon screening the reaction conditions for the MBH reaction with acrylonitrile (see SI 5.4 for details). Nonetheless, in both cases, we observed that optimal reaction conditions were not correlated with the highest number of equivalents of reagents and base (entries 10 and 17). As a matter of fact, a sharp decrease in conversion is observed upon increasing amounts of olefins and DABCO, especially in the case of ethyl acrylate. This prompted us to evaluate mechanistic aspects in more details.

Starting from suboptimal reaction conditions, DNA-aldehyde (0.33 mM), DABCO (0.33 M), and **2** (0.33 M), we initiated this endeavor by analyzing the influence of substrate concentration on the conversion and, albeit an initial inverse correlation can be observed in the first hours, no discernible difference among each initial substrate concentration is marked (see SI 5.5 for details).

However, a slight decrease in conversion is clearly observed after 16 h with the lowest **1a** concentration (Figure 2), potentially suggesting a puzzling absence of equilibrium. To address it, a series of experiments with varying DABCO

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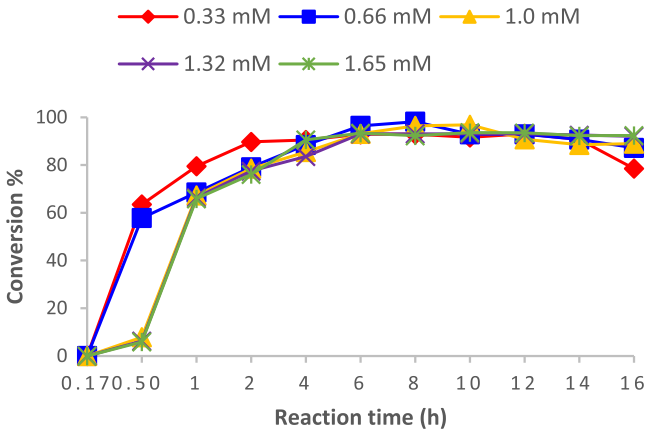

Figure 2. MBH reaction of **2** with various concentration of **1a**. DNA-aldehyde (0.33−1.65 mM), DABCO (0.33 M), olefin (0.33 M), CH_3CN/H_2O = 2/1, 25 °C, 16 h.

concentrations but constant **1a** and ethyl acrylate **2** concentrations (0.33 M) were conducted (Figure 3).

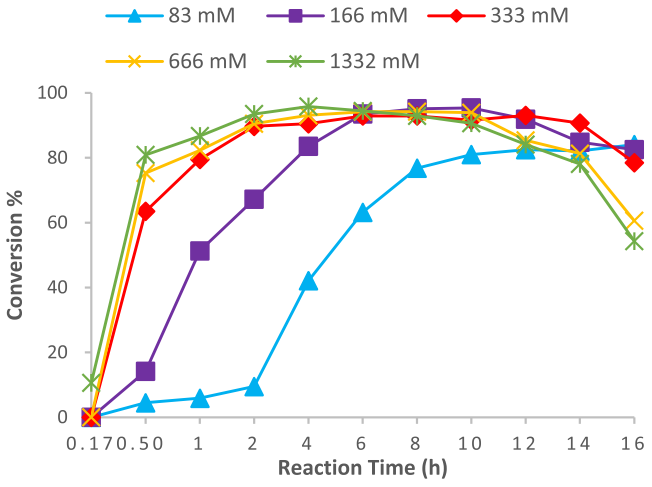

Figure 3. MBH reaction of **2** with **1a** and various DABCO concentration. DNA-aldehyde (0.33 mM), DABCO (variable), olefin (0.33 M), CH_3CN/H_2O = 2/1, 25 °C, 16 h.

After 30 min, a clear positive correlation between reaction rate and DABCO concentration can be observed. However, after 8 h, the conversion rate of the reaction with the highest concentration of DABCO gradually decreased. In parallel, upon monitoring starting material consumption, we observed a concomitant initial decrease in substrate, reaching a low plateau up to 8 h, followed by an increase up to well-detectable levels (Figure 4).

Nonetheless, we view the on-DNA MBH mechanism as a special opportunity for new mechanistic study (Figure 5), since there are significant differences among on-DNA and off-DNA reactions and obvious unusual characteristics of on-DNA MBH reactions. Based on previous studies, the MBH reaction can reach nearly 100% conversion in 4 to 6 h when DABCO concentration is more than 166 mM (with DNA-Aldehyde (0.33 mM), olefin (0.33 M), CH_3CN/H_2O = 2/1, 25 °C). Given that the MBH reaction is carried out in aqueous solution, the aldol reaction likely follows Hill and Isaacs' proposed route to create B.[43] With a large excess amount of DABCO, Michael addition of product B with DABCO yields C

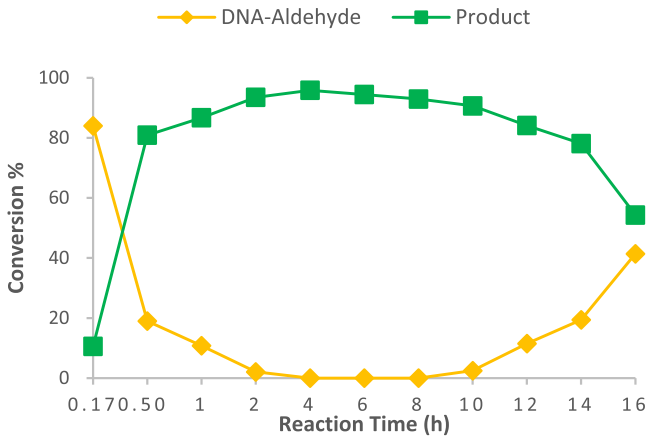

Figure 4. Time course studies of the MBH reaction. DNA-aldehyde (**1a**, 0.33 mM), DABCO (1.33 M), olefin (**2**, 0.33 M), CH_3CN/H_2O = 2/1, 25 °C, 0 to 16 h.

and/or D. C can decompose through the retro-aldol reaction and elimination to starting materials via Route a, while D collapses through the retro-aldol reaction and elimination to starting materials via Route c. Notwithstanding, C or D could convert to A via elimination and the reaction cycle would restart, establishing a dynamic equilibrium. However, our finding revealed that the reaction progress was time-dependent and was not equilibrated. Hence, a scavenger likely converts the product into an intermediate and prevents it from reacting further with DABCO and ethyl acrylate.

Although the anticorrelation of consumption of starting material and conversion of the desired products were well characterized, we were not able to observe the apparition of products C and D using LCMS analysis, where we could only observe the starting material A being reformed. Consequently, one possible explanation is that pseudostable C and/or D acts as a drain for B and were then completely and rapidly decomposed into A during LCMS analysis, with heating over the chromatography column or the electrospray ionization process. Our second hypothesis is predicated on the irreversible hydrolysis of ethyl acrylate **2**, which has a stronger basis for revealing the mechanism of the reaction. When MBH reactions were conducted with 1.33 M DABCO (with DNA-Aldehyde (0.33 mM), olefin (0.33 M), CH_3CN/H_2O = 2/1, 25 °C), it was more likely that the MBH reaction progressed while ethyl acrylate **2** hydrolyzed to acrylic acid E. Since E could not undergo the MBH reaction, the equilibrium was hence disrupted after Stage 1 equilibrium. The forward reaction to produce MBH adducts predominated because it was significantly faster during Stage 1, whereas the reverse reaction prevailed during Stage 2, driven by the reduction of the ethyl acrylate **2** concentration.

These findings convinced us that the on-DNA reaction is indeed reversible in the case of acrylates under a high concentration of DABCO, although this two-phase process occurrence was difficult to comprehend. Notably, it proved that DABCO seemed to play a dual role in the process, facilitating the conversion in the first place, and once the conversion reached its maximum, accelerating the decomposition of the desired product and converting it back into the starting material. These results compelled us to investigate the mechanism further.

In recent years, the mechanism of MBH reaction has raised considerable interest.[49−59] MBH reaction process is commonly

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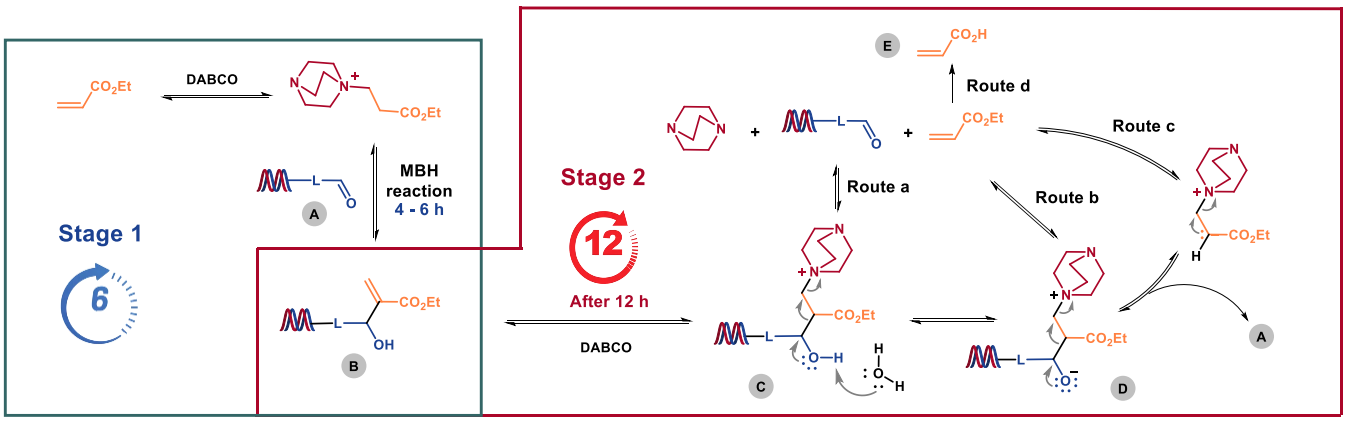
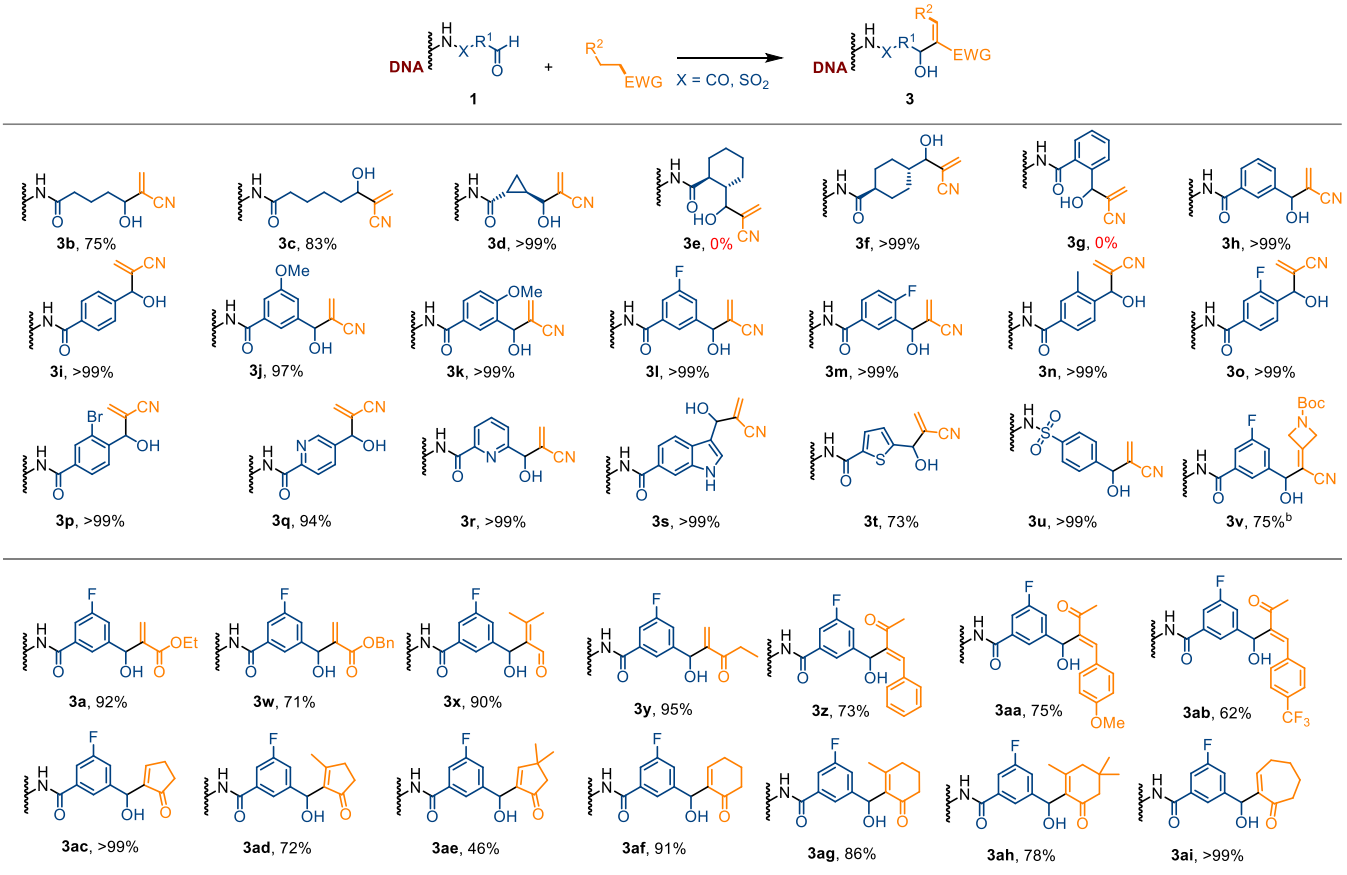


Figure 5. Plausible mechanism of the on-DNA MBH reaction and its reverse process.

Table 2. Morita-Baylis-Hillman Reaction with Various DNA-Conjugate Aldehydes and Michael Acceptors[a]



[a]Standard reaction conditions: **1** (10.0 nmol, 5.0 mM in H_2O, 1.0 equiv, 2.0 μL, 1.65 mM final concentration), Michael acceptors (200 equiv, 1.00 M in CH_3CN, 2.0 μL, 333.3 mM final concentration), DABCO (200 equiv, 1.00 M in CH_3CN, 2.0 μL, 333.3 mM final concentration), 25 °C, 16 h. Conversions were determined by LC-MS. [b]Reaction temperature was 60 °C.

accepted as consisting in an "addition" step by an activating nucleophile, followed by a carbon−carbon bond-forming "aldol reaction" step, and an "elimination" step to afford the product.[57]

To offer proof for the second scenario, acrylonitrile, considerably less prone to degradation, was utilized in the MBH reaction under identical conditions. After 16 h, there was negligible reversal reaction (<5% after 16 h). In addition, the concentration variations of **2** and E in the reaction mixture of the MBH reaction were monitored using ^{1}H NMR studies under conditions identical to those of the on-DNA reaction without DNA. The results demonstrated that ester hydrolysis occurred during the reaction and generated inactive acrylic acid. In the meantime, the lower concentration of acrylate **2** weakened the conversion to the MBH adduct (see SI 5.10–5.12 for details).

Driven by these mechanistic findings and by our initial reaction conditions optimization, we then moved to a more thorough analysis of the reaction scope. Interestingly, although our mechanistic investigation revealed the existence of a

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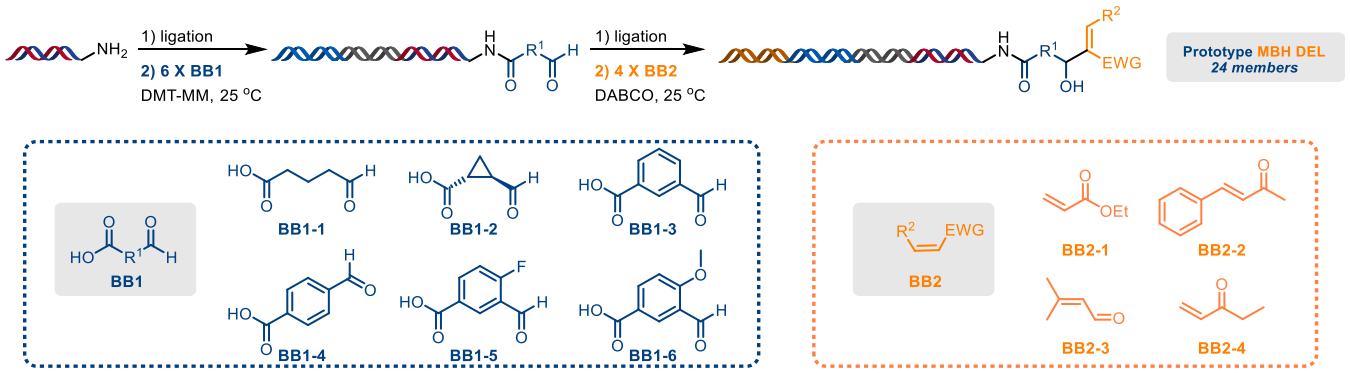

Figure 6. Prototype DEL synthesis.

nonequilibrated process based on the dual role of DACBO and the potential acrylate hydrolysis, our optimal reaction conditions, increasing substrate concentrations while limiting the amount of base and therefore decreasing the likeness for the reaction to tip over its reversal, we confidently move to the substrate scope assessment, starting with acrylonitrile as the electrophile. Initial explorations rapidly revealed that the transformation is compatible with a variety of aldehyde substrates (Table 2), including both acyclic aliphatic and cyclic aliphatic carboxyl aldehydes (**3b**−**3f**, except steric hindered **3e**), and a vast majority of aromatic carboxyl aldehydes, as well as heterocyclic aldehydes.

The influence of steric factors was clearly identified on aromatic benzyl carboxylic aldehydes as the *ortho*-isomer (**3g**) failed to convert while *meta* and *para*-isomers (**3h**, **3i**) worked particularly smoothly. No influence of electronic factors was however observed as substrates with both electro-donating (**3j**, 97%; **3k**, > 99%) and electron-withdrawing groups (**3l**, > 99%; **3m**, > 99%) were tolerated on 3-formylbenzamide cores. Similarly, 4-formylbenzamide substrates with both electro-donating (**3n**, > 99%) and electron-withdrawing groups (**3o**, > 99%; **3p**, > 99%) led to complete conversions under the aforementioned optimal conditions. Encouraged by these results, we shifted our focus to heterocyclic substrates. To our delight, the reaction thrived with pyridinyl carboxyl aldehydes (**3q**, 94%; **3r**, > 99%), indole carboxyl aldehydes (**3s**, > 99%) and thiophene substrate (**3t**, 73%), respectively. In addition to the classic amide linkage to DNA, the substrate with sulfonamide link behaved perfectly (**3u**, > 99%). Using 3-fluoro-5-formyl benzamide **1a**, ^{t}Butyl 3-(cyanomethylene) azetidine-1-carboxylate was reacted smoothly and proved that more structurally complex ethylene cyanides are applicable to the methodology (**3v**, 75%).

Next, we explored the versatility of the approach by varying the types of Michael acceptors. Benzyl acrylate was proved to react smoothly with our model substrate (**3w**, 71%), as well as 3-methylbut-2-enal and pent-1-en-3-one (**3x**, 90%; **3y**, 95%). Remarkably, the optimal conditions proved their versatility, as no undesired reversal was observed with this new acrylate. While switching to arylated Michael acceptors, we did observe a moderate electronic influence as (*E*)-4-phenylbut-3-en-2-one and its *para*-substituted counterparts were generally reactive (**3z**, **3aa**, **3ab**), albeit slightly lower conversion presented with the electron-withdrawing group on the *para*-phenyl (**3ab**). We pursued the study with cyclic Michael acceptors and observed remarkable, yet surprising, reactivity with cyclopent-2-en-1-one (**3ac**). Indeed, substituted 3-methylcyclopent-2-en-1-one and 4,4-dimethylcyclopent-2-en-1-one were transformed much less effectively. However, this drastic drop in reactivity may likely be attributed to steric hindrance (**3ad**, 72%; **3ae**, 46%). Moving on to six-membered rings with cyclohex-2-en-1-one, we observed an unsurprising lower yet sensible influence of steric effects as its methylated counterparts 3-methylcyclohex-2-en-1-one and 3,5,5-trimethylcyclohex-2-en-1-one (**3af**, **3ag**, **3ah**). An additional ring expansion, with cyclohept-2-en-1-one and its excellent conversion (**3ai**), demonstrated that the ring size of Michael acceptors is less relevant to conversion than the steric hindrance, which proved to be a crucial factor for rich DEL compound diversity.

Although a strict comparative study with our results and previously reported MBH examples can be biased, due to the "on-DNA condition" features, our advances are noteworthy: with overall high conversion, relatively short reaction times, and the undeniable presence of an aqueous reaction medium truly allow this study to stand out from previous off-DNA endeavors most importantly considering the inherent existence of an undesired nonequilibration phenomenon. Moreover, most of the formed products are inherently novel.

Interestingly, the MBH reaction can be scaled up easily. Compound **1n** was reacted with acrylonitrile to yield the desired product (**3o**) in >99% at 50 nmol scale, comparable to conversion at a 10 nmol scale. Hence, the implementation of this methodology in routine DEL synthesis should not suffer from any scale factors.

To further illustrate the efficacy of the methodology in the library synthesis protocol, we embarked on the design of a prototype DEL (Figure 6). The planned DEL is composed of 2 cycles of synthesis. After ligation with unique codons at Cycle 1, each encoded on-DNA starting materials were coupled with six aliphatic or aromatic acid-aldehyde bifunctional reagents through acylation. The pooled Cycle 1 products were then subjected to ligation with tag B followed by MBH reaction with 4 different Michael acceptors as Cycle 2 reagents, under our optimized conditions to yield a pool of 24 compounds after HPLC purification.

Next, we assessed DNA integrity upon the on-DNA MBH reaction through real-time quantitative polymerase chain reaction (qPCR) studies. The results indicated that no appreciable DNA damage from the standard reaction conditions could be observed (see SI 10.6 for details). Moreover, a capped substrate with no predicted reactivity was exposed to the standard reaction conditions as a control experiment, and the results demonstrated that the DNA oligo conjugate remained intact throughout the reaction process, thereby further validating the integrity of the encoding DNA tags (see SI 8.2 for details).

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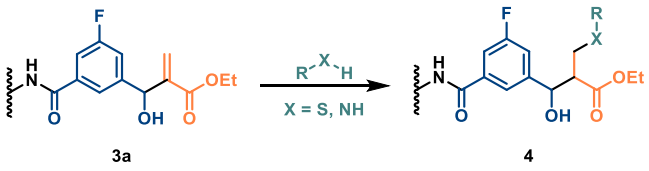
Next-generation sequencing (NGS) decoding results demonstrated that the total decodability rate with a perfect match between the prototype DEL and DNA-only library is comparable (see SI table S5 for details). Each codon is displayed after the DEL synthesis. Similar mismatching rates of 1 bp sequences are observed in prototype DNA-only and DEL libraries. Based on our experience, DNA damage during the MBH reaction is among the lowest when compared to other reactions such as the Sonogashira reaction, Suzuki coupling, Boc group deprotection, amindation, Kinugasa reaction, Glaser reaction, and acylation (see SI 10.8).

Additional experiments reveal that the on-DNA MBH reaction devised works well with longer DNA oligos (see SI 5.13 for details).

The on-DNA MBH adduct has been confirmed to be stable under the selection conditions for covalent-binging by following the method described in the literature (see SI Section 12, page S289 for more information).[60] Further application for Michael addition with free thiol or amine can undergo the anticipated addition with free thiol to demonstrate the covalent binding utility of the products for future DEL selection (Figure 7). In addition, it validates the structure of MBH adducts (SI for Figures S135 and S136 for details).



3a 4

Figure 7. Further chemical transformation exploration of MBH adducts.

In conclusion, we have developed a robust and novel bioconjugation strategy with an on-DNA Morita-Baylis-Hillman reaction for DEL syntheses. Notably, this methodology should be envisioned as an efficient and versatile method for the creation of POI covalent-binding capable compounds. To the best of our knowledge, this new on-DNA protocol truly brings a new paradigm to light, as MBH reactions and its variants, although synthetically powerful, often suffer from inherent impediments. This methodology, along with its associated mechanistic study, demonstrated the possibility to carry out this key transformation under mild conditions with high conversions and a broad functional group tolerance while preventing intrinsic undesired reactivities. Moreover, it surely widens the field of possibility by providing access to yet uncharted chemical space in DEL, with promising new opportunities for drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.3c00138.

Experimental details and procedures, optimization studies, and spectral data for all on-DNA compounds (PDF)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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