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A DNA-compatible [3+2] Nitrone–Olefin Cycloaddition Suitable for DEL Syntheses

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Abstract

The limited scope of DNA-compatible chemistry restricts the types of chemical features that can be incorporated into DNA-encoded libraries (DELs). Here, a method to synthesize DNA-conjugated polycyclic isoxazolidines *via* a [3+2] nitrone–olefin cycloaddition is described. The reaction is compatible with many olefin-containing substrates and diverse *N*-alkylhydroxylamines. The ability to perform subsequent DNA ligation and PCR amplification was also confirmed. This methodology facilitates the synthesis of DELs containing topographically complex compounds with underexplored chemical features.

Graphical Abstract

DNA-encoded libraries (DELs) of small molecules can facilitate the identification of protein “binders”^{1,2} in affinity-based biochemical assays.^{3–6} DELs combine synthetic organic chemistry and DNA barcoding to generate libraries of compounds that are each covalently

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Author Contributions

C.J.G. and S.L.S. designed the research and wrote the manuscript. C.J.G., Z.Y., and M.S. performed off-DNA chemistry. C.J.G. performed all on-DNA chemistry, molecular biology experiments, and data analysis. All authors have edited the manuscript and have given approval to the final version.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed methods for all chemical and biological procedures, characterization data (including NMR spectra) for off-DNA synthetic intermediates, UPLC-MS data for optimized on-DNA chemical reactions, Supporting Figures and Tables, and additional details regarding reagents and instruments used (PDF)

S.L.S. is a member of the Board of Directors of the Genomics Institute of the Novartis Research Foundation (“GNF”); a shareholder and member of the Board of Directors of Jnana Therapeutics; a shareholder of Forma Therapeutics; a shareholder of and adviser to Decibel Therapeutics and Eikonizo Therapeutics; an adviser to Eisai, Inc., the Ono Pharma Foundation, and F-Prime Capital Partners; and a Novartis Faculty Scholar.

linked to a unique, amplifiable tag that records the synthetic reactions from which individual compounds derive.⁷ DELs are most often used to identify molecules that engage an immobilized protein target.⁸ This strategy has thus far yielded binders of therapeutically relevant proteins such as kinases,⁹ phosphatases,¹⁰ G-protein coupled receptors (GPCRs),¹¹ and others.¹²

It is possible that the full potential of DEL-mediated target-based screening has not been realized because, despite recent advances in DNA-compatible chemistry,^{13–16} the types of structural features found in DELs remain limited.¹⁷ To generate libraries, DNA-encoded compounds are often synthesized in parallel with their barcodes using split–pool synthesis. The presence of DNA and water during synthesis, however, restricts the chemistry that may be used to build DELs. Many robust and commonly used transformations (*e.g.* cross-couplings, amidations, and S_NAr reactions)¹⁸ tend to produce compounds rich in sp²-hybridized carbons. Therefore, significant gaps exist in the types of molecular architectures found in DELs.¹⁹ In an effort to address these shortcomings, a novel, open-source collaboration involving a small group of scientists at the Novartis Institutes for BioMedical Research and the Broad Institute was recently established. The work reported here describes an early example of this collaboration.

We identified the intramolecular [3+2] nitron–olefin cycloaddition as a transformation that can fill some of the structural gaps in current DELs. Earlier reports have shown this reaction to be both water- and DNA-compatible,^{20,21} though it has never been used for DEL synthesis, and previous DNA-compatible examples were performed using DNA-templated synthesis with activated olefins and pre-formed nitrones.²⁰

From a structural standpoint, this reaction converts sp²-rich starting materials to sp³-rich isoxazolidines containing at least two stereogenic elements. The isoxazolidine motif is rarely found in DELs, but it resides in several alkaloid natural products and other bioactive compounds.^{22,23} Furthermore, unlike most other ring-forming reactions (*e.g.* ring-closing metathesis), it can introduce appendage diversity *via* condensation of *N*-alkylhydroxylamines with aldehydes or ketones to form nitrones *in situ*. The ability to perform ring closure and appendage diversification simultaneously is appealing because split–pool synthesis often makes post-reaction purification challenging. Therefore, DEL pathways should be limited to a handful of steps to mitigate buildup of DNA damage and unwanted by-products, among other factors.²⁴

We first synthesized on-DNA salicylaldehyde derivative **1** and treated it with methylhydroxylamine; we used the double-stranded DNA “headpiece” commonly used in split–pool DEL synthesis.⁶ Our initial experiments suggested that conversion to the desired isoxazolidine **2** could be increased by raising the reaction temperature (Table 1; entries 1–2). Performing the reaction at 100 °C, however, resulted in decomposition of the DNA barcode *via* depurination that was detectable by UPLC-MS analysis (Table 1; entry 3). The use of organic base (Table 1; entry 4) or additional equivalents of hydroxylamine (Table 1; entry 5) resulted in low conversion and substantial by-product formation. While high conversions could be achieved in 4 h at 90 °C in basic phosphate buffer (Table 1; entry 6), even higher conversions were observed in acidic phosphate buffer (Table 1; entry 7). However, the

substrate was more prone to depurination in acidic conditions (Table 1; entry 8), so milder buffering systems were explored (Table 1; entries 9–12). Ultimately, eight volumes of 0.1 M NaOAc buffer yielded the highest conversion (Table 1; entry 12). The *cis* ring fusion of **2** was confirmed *via* NMR-based characterization of an off-DNA analog (see Supporting Information for details).

We then studied the compatibility of this transformation with additional hydroxylamines; the reaction conditions were modified slightly to account for the increased complexity and steric bulk of the nitron intermediates (Table S1). While methyl-, ethyl-, and *n*-hexylhydroxylamine afforded clean conversions to the desired isoxazolidines (Table 2; entries 1–3), by-products were observed when **1** was treated with *i*PrNHOH (Table 2; entry 4). We identified one species as the obligatory nitron intermediate; it both had the same mass as the desired isoxazolidine product and appeared to convert to it over time (Figure S1). The other species, which was independent of the hydroxylamine in the reaction, was an oxime-containing product likely derived from interception of the nitron intermediate by an exogenous nucleophile. Both products are consistent with the increased steric bulk of the nitron slowing the rate of the intramolecular cyclization.

The steric bulk of *t*BuNHOH (Table 2; entry 5) hindered both the [3+2] cycloaddition and condensation to the nitron; however, increasing the amount of hydroxylamine 4-fold (Table 2; entry 6) yielded substantially higher isoxazolidine formation. Cyclopentyl- and cyclohexylhydroxylamine achieved 88% and 82% conversion, respectively (Table 2; entries 7–8), both of which were higher than *i*PrNHOH (77%). These differences in reactivity underscore the outsized impact of steric effects upon the success of the reaction.

We then tested hydroxylamines containing functional groups other than saturated hydrocarbons. Benzyl-containing hydroxylamines were very successful (Table 2; entries 9–10), but an electrophilic 2-pyridine derivative yielded high amounts of oxime (Table 2; entry 11). Both free and protected amines are compatible with the reaction, though we observed some phthalimide deprotection (Table 2; entries 12–13). *N*-hydroxy-L-ornithine generated high levels of oxime (Table 2; entry 14), potentially *via* an intramolecular 5-*exo-tet* cyclization to generate the on-DNA oxime and the presumed ornithine-derived proline. A bulky sulfone-containing reagent initially afforded low consumption of **1**, but additional hydroxylamine furnished excellent results (Table 2; entries 15–16). Finally, treatment with aromatic hydroxylamines resulted in no isoxazolidine formation (Table 2; entries 17–18).

Next, we studied the effect of olefin substitution. Ten additional on-DNA substrates (**3–12**) were synthesized, and each was initially tested with three hydroxylamines (Figure 1). Unsurprisingly, due to the sensitivity of the reaction to steric effects (see Table 2; entries 1–8), all experiments with prenylated substrate **3** resulted in poor isoxazolidine formation.

To determine if favorable electronics could outweigh the penalty of increased steric bulk, we synthesized DNA-linked substrates featuring a variety of electron-withdrawing groups on the reactive olefin (**4–12**; Figure 1). For each substrate, significant product formation was observed after 8 h at just 40 °C. Even under these mild conditions, however, saponification was observed with esters **4–7**. Of these four substrates, isopropyl ester **6** exhibited the lowest

susceptibility to hydrolysis. Substrates **8–10** often yielded high conversions and simple reaction profiles. Though treatment of **8** and **10** with *i*PrNHOH resulted in low isoxazolidine formation, in each case the only other major product was the nitron intermediate. Nitrile **11**, on the other hand, always generated a complex mixture of products that could not be easily identified by UPLC-MS analysis alone. Finally, sulfone **12** afforded moderate isoxazolidine formation with all three hydroxylamines.

Having seen that activated olefins accelerate on-DNA cyclizations dramatically, we studied the hydroxylamine scope further with substrates **6** and **8–10** (Table 3). We tailored the reaction parameters for each substrate to yield maximal isoxazolidine formation under the mildest possible conditions; **9** achieved very similar results with the harsher conditions associated with **8** and **10** (Table S2), which suggests **8–10** (or derivatives) could be pooled for DEL synthesis.

Using these optimized conditions, treatment with methyl- and *n*-alkylhydroxylamines largely resulted in excellent isoxazolidine formation (Table 3; entries 1–12). Experiments with ester **6** were less successful due to incomplete consumption of starting material and the formation of saponification by-products; this trend continued throughout the study. All substrates yielded slightly lower conversions with *i*PrNHOH (Table 3; entries 13–16). Lower conversions still were seen with *t*BuNHOH (Table 3; entries 17–20) due to sluggish nitron formation and cyclization. Cyclopentylhydroxylamine behaved similarly to *i*PrNHOH (Table 3; entries 21–24), but reactions with the bulkier cyclohexyl reagent were superior due to decreased oxime formation (Table 3; entries 25–28). Next, reactions with benzyl-containing hydroxylamines proceeded smoothly (Table 3; entries 29–36). Most experiments with the pyridine-containing reagent, however, suffered from high rates of oxime formation, though **9** was sufficiently reactive to undergo cyclization before this side-reaction could occur (Table 3; entries 37–40). Both free and phthalimide-protected amines were again tolerated (Table 3; entries 41–48), and minimal deprotection was observed under these milder conditions. *N*-hydroxy-L-ornithine generally furnished high amounts of oxime by-product and complex reaction profiles (Table 3; entries 49–52), but the sulfone-containing hydroxylamine yielded excellent results (Table 3; entries 53–56). We did not observe products of conjugate addition—which is a known DNA-compatible reaction²⁵—in any experiment.

The compatibility of this transformation with several commonly used functional groups in DNA-compatible chemistry—free and protected amines, carboxylic acids, and aryl halides—highlights its utility for DEL design. In strategically designed on-DNA chemical pathways, these chemical handles (*e.g.* the carboxylic acid of **8**) would be functionalized either before or after cyclization to enable the construction of large, combinatorial DELs containing rare structural features.

We have shown that this methodology can achieve high yields with a variety of substrates. But to be suitable for DEL synthesis, it must also leave the DNA barcode capable of undergoing enzymatic DNA ligation and PCR amplification.²⁶ To assess post-reaction DNA integrity (Figure 2), we performed cycloadditions with substrates **1**, **9**, and **10** using each scaffold's optimized reaction conditions. Then, we ligated an oligonucleotide to the reaction products to generate a barcode comparable in size to those found in a 3-cycle DEL. Finally,

we successfully amplified all three barcodes by PCR and detected no loss of informational integrity (Figures S2–S5).

In conclusion, we have developed a DNA-compatible [3+2] nitron–olefin cycloaddition capable of generating polycyclic isoxazolidines. This transformation routinely eclipses 80% product formation with a variety of *N*-alkylhydroxylamines and olefin-containing substrates, which makes it appealing for DEL construction. We also confirmed that three sets of our optimized reaction conditions did not damage the DNA barcode or prevent subsequent ligation and amplification steps required for DEL synthesis.

DELs that incorporate this transformation may begin to fill some of the structural—and perhaps functional—gaps left by current on- and off-DNA small-molecule libraries. Efforts to leverage this reaction most effectively for DEL synthesis are ongoing, and we aim to describe these at a later date.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Conversions to isoxazolidines for on-DNA substrates **3–12**. Reactions carried out with 40 equiv hydroxylamine (50 mM in DMSO) in 1.0 M NaOAc aq buffer (pH 5.0) for 8 h at 40 °C. ^a35 equiv hydroxylamine for 2 h at 40 °C then 6 h at 90 °C.

Figure 2.

Schematic for three-step assessment of DNA integrity. DNA attached to cycloaddition substrates **1**, **9**, and **10** successfully underwent DNA ligation and PCR amplification after [3+2] nitron–olefin cycloaddition.

Table 1.

Initial optimization of on-DNA [3+2] nitron-olefin cycloaddition

entry	time (h)	temp (°C)	MeNHOH (equiv)	buffer	buffer vols ^g	2 (%) ^h
1	12	70	25	Na ₂ HPO ₄ ^a	2	70
2	12	90	25	Na ₂ HPO ₄ ^a	2	88
3	12	100	25	Na ₂ HPO ₄ ^a	2	84 ^f
4	12	70	25	pyrrolidine ^b	0.1	54
5	12	90	100	Na ₂ HPO ₄ ^a	2	64
6	4	90	25	Na ₂ HPO ₄ ^a	0.5	86
7	4	90	25	NaH ₂ PO ₄ ^c	0.1	93
8	12	90	25	NaH ₂ PO ₄ ^c	0.5	81 ^f
9	4	90	25	NaH ₂ PO ₄ ^d	2	90
10	4	90	25	NaH ₂ PO ₄ ^d	8	71
11	4	90	25	NaOAc ^e	2	91
12	4	90	25	NaOAc ^e	8	>98

^a 1.0 M aq buffer (pH 9.2).^b 0.2 M DMA solution.^c 1.0 M aq buffer (pH 4.2).^d 0.1 M aq buffer (pH 5.9).^e 0.1 M aq buffer (pH 5.0).^f Significant DNA damage observed.^g Compared to amount of **1** stock solution (0.8 mM) added.

^hDetermined by UPLC-MS.

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Table 2.

Scope of hydroxylamine with substrate 1

Reaction scheme: Substrate 1 reacts with hydroxylamine in NaOAc buffer with heat (Δ) to form isoxazolidine, nitron, or oxime products.

entry	hydroxylamine	isoxazolidine (%) ^c	nitron (%) ^c	oxime (%) ^c
1		>98	0	0
2		>98	0	0
3		94	0	0
4		77	13	10
5		38	9	0
6 ^a		77	15	0
7		88	0	12
8		82	7	11
9		>98	0	0
10		96	3	0
11		37	0	63
12		>98	0	0
13		87 ^b	0	0
14		31	0	69
15		46	0	2
16 ^a		95	0	5
17		0	0	0
18		0	0	55

Reactions carried out with 35 equiv hydroxylamine (50 mM in DMSO) in 0.1 M NaOAc aq buffer (pH 5.0) for 8 h (2 h at 40 °C then 6 h at 90 °C). ^a140 equiv hydroxylamine. ^b50% phthalimide, 37% free amine. ^cDetermined by UPLC-MS.

Table 3.

Scope of hydroxylamine with **6** and **8–10**

entry	hydroxylamine	R ¹	isoxazolidine (%) ^c	entry	hydroxylamine	R ¹	isoxazolidine (%) ^c
1 ^a		CO ₂ iPr (6)	67 ^a	29 ^a		CO ₂ iPr (6)	52 ^a
2 ^b		CO ₂ H (8)	>98 ^a	30 ^b	Ph-CH ₂ -OH	CO ₂ H (8)	90 ^a
3 ^c	Me-CH ₂ -OH	CF ₃ (9)	98 ^a	31 ^c		CF ₃ (9)	92 ^a
4 ^b		CONHiPr (10)	96 ^a	32 ^b		CONHiPr (10)	>98 ^a
5 ^a		CO ₂ iPr (6)	62 ^a	33 ^a		CO ₂ iPr (6)	63 ^a
6 ^b		CO ₂ H (8)	>98 ^a	34 ^b		CO ₂ H (8)	88 ^a
7 ^c	Me-CH ₂ -OH	CF ₃ (9)	96 ^a	35 ^c		CF ₃ (9)	91 ^a
8 ^b		CONHiPr (10)	97 ^a	36 ^b		CONHiPr (10)	90 ^a
9 ^a		CO ₂ iPr (6)	59 ^a	37 ^a		CO ₂ iPr (6)	36 ^a
10 ^b		CO ₂ H (8)	94 ^a	38 ^b		CO ₂ H (8)	N.D.
11 ^c		CF ₃ (9)	97 ^a	39 ^c		CF ₃ (9)	>98 ^a
12 ^b		CONHiPr (10)	>98 ^a	40 ^b		CONHiPr (10)	18 ^a
13 ^a		CO ₂ iPr (6)	54 ^a	41 ^a		CO ₂ iPr (6)	52 ^a
14 ^b		CO ₂ H (8)	85 ^a	42 ^b		CO ₂ H (8)	95 ^a
15 ^c	Me-CH ₂ -OH	CF ₃ (9)	84 ^a	43 ^c		CF ₃ (9)	97 ^a
16 ^b		CONHiPr (10)	84 ^a	44 ^b		CONHiPr (10)	>98 ^a
17 ^a		CO ₂ iPr (6)	52 ^d	45 ^a		CO ₂ iPr (6)	58 ^a
18 ^b		CO ₂ H (8)	50 ^a	46 ^b		CO ₂ H (8)	79 ^a
19 ^c		CF ₃ (9)	18 ^a	47 ^c		CF ₃ (9)	>98 ^a
20 ^b		CONHiPr (10)	51 ^a	48 ^b		CONHiPr (10)	95 ^a
21 ^a		CO ₂ iPr (6)	50 ^a	49 ^a		CO ₂ iPr (6)	N.D.
22 ^b		CO ₂ H (8)	89 ^a	50 ^b		CO ₂ H (8)	36 ^a
23 ^c		CF ₃ (9)	81 ^a	51 ^c		CF ₃ (9)	31 ^a
24 ^b		CONHiPr (10)	86 ^a	52 ^b		CONHiPr (10)	37 ^a
25 ^a		CO ₂ iPr (6)	56 ^a	53 ^a		CO ₂ iPr (6)	60 ^a
26 ^b		CO ₂ H (8)	95 ^a	54 ^b		CO ₂ H (8)	>98 ^a
27 ^c		CF ₃ (9)	93 ^a	55 ^c		CF ₃ (9)	90 ^a
28 ^b		CONHiPr (10)	93 ^a	56 ^b		CONHiPr (10)	>98 ^a

Reaction conditions: 100 equiv hydroxylamine (50 mM in DMSO) in 1.0 M NaOAc aq buffer (pH 5.0), ^a8 h at 50 °C, ^b8 h at 60 °C, ^c4 h at 40 °C, ^d50 equiv hydroxylamine. ^eDetermined by UPLC-MS; green = 80–100%, orange = 50–79%, red = 0–49%; N.D. = not determined