

Homogeneous and Functional Group Tolerant Ring-Closing Metathesis for DNA-Encoded Chemical Libraries

Olivier B. C. Monty,[§] Pranavanand Nyshadham, Kurt M. Bohren, Murugesan Palaniappan, Martin M. Matzuk, Damian W. Young,* and Nicholas Simmons*^{*,§}



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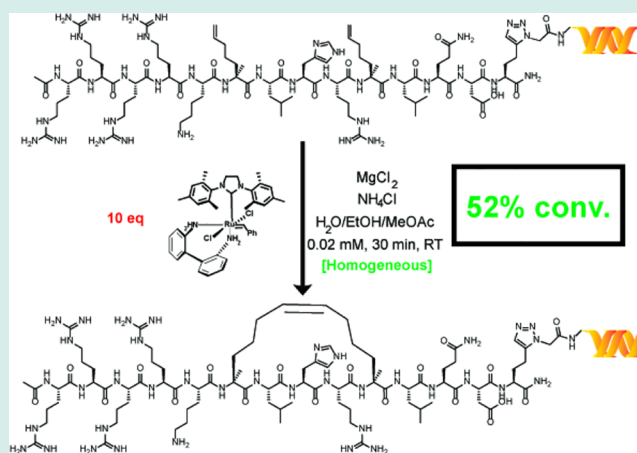


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ABSTRACT: Reaction heterogeneity, poor pH control, and catalyst decomposition in the ring-closing metathesis (RCM) of DNA–chemical conjugates lead to poor yields of the cyclized products. Herein we address these issues with a RCM reaction system that includes a novel aqueous solvent combination to enable reaction homogeneity, an acidic buffer system which masks traditionally problematic functional groups, and a decomposition-resistant catalyst which maximizes conversion to the cyclized product. Additionally, we provide a systematic study of the substrate scope of the on-DNA RCM reaction, a demonstration of its applicability to a single-substrate DNA-encoded chemical library that includes sequencing analysis, and the first successful stapling of an unprotected on-DNA [i, i+4] peptide.



KEYWORDS: DNA-encoded chemistry, DNA-encoded libraries, homogeneous ring-closing metathesis, functional group-tolerant ring-closing metathesis, stapled peptides

INTRODUCTION

Since the conception of DNA-encoded combinatorial chemical libraries by Brenner and Lerner,¹ many variations of DNA-encoded chemical screening technologies have been developed to aid drug discovery efforts.² Advancements both in the production of large quantities of synthetic DNA oligomers³ and in high-throughput DNA-sequencing methods⁴ now allow for rapid and cost-effective screens of vast DNA-encoded chemical library (DECL) collections against biological targets.⁵ DECLs are single-pot chemical libraries consisting of compounds that each possess a covalently attached, unique DNA sequence “barcode”, which enables identification of binder compounds by DNA sequencing after multiplexed target-based screens. Compared to high-throughput compound collections and screens, DECLs are relatively inexpensive to prepare and use⁶ and, most importantly, offer the opportunity for deeper exploration of chemical space⁵ enabled by unprecedented numbers of compounds per DECL—millions to trillions compared to only hundreds of thousands per HTS chemical library.⁷ Additionally, DECLs have provided starting points for the development of several clinical candidates^{6,8} and are considered by many to have become one of the pillars of drug discovery.⁹ However, while large numbers are the main advantage of DECLs, the chemical diversity achievable under its umbrella is limited by the number of effective DNA-compatible reactions. Currently, only a limited set of

DNA-compatible, solution-phase chemical reactions has been reported,^{10–18} and expanding the repertoire of chemical reactions to more effectively sample chemical space is a major goal within this area.

Within the compendium of synthetic methodologies, the ring-closing metathesis (RCM) reaction has become a mainstay for the construction of organic molecules spanning a wide range of structural diversity and complexity. Accordingly, it has been applied across medicinal, natural product, and diversity-oriented synthetic endeavors.^{19–22} The widespread popularity of RCM is grounded in several reasons: its broad and well-studied functional group and substrate tolerance, the commercial availability of a wide array of air-stable, tunable Ru-based catalysts,^{23,24} its successful application in aqueous media,^{25,26} and its relevance to the production of drug-like compounds^{27,28} as well as novel molecular frameworks for the probing of unexplored chemical space.²² DECL productions are based on combinatorial chemistry, and the RCM reaction would therefore be a significant enhancement to the technology. Stimulated by our interest in

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combining chemical diversity and large numbers for drug discovery, we became interested in the application of RCM to the production of DECLs. Our work was not done in a vacuum, however, and the precedents upon which we built need mentioning.

The modification of proteins using the cross-metathesis (CM) reaction²⁹ has been demonstrated in aqueous *tert*-butanol, using a high excess (10,000 equiv) of MgCl₂ as a Lewis acidic masking agent. Additionally, RCM-stapling of unprotected peptides has been achieved in water using the water-soluble AquaMet catalyst, also in the presence of MgCl₂ (400 equiv).³⁰ The Mg²⁺ ion is believed to act as a mild Lewis acid, masking coordinating functional groups typically prevalent in biomolecules.^{29,31} Finally Lu et al.³² reported the use of a third generation Grubbs catalyst/MgCl₂/aq. *tert*-butanol system for the on-DNA RCM and cross metathesis (CM) reactions. While the latter study established that RCM could be achieved on DNA–chemical conjugates, the reported conditions were not adequate, in our hands, for the use of RCM in a DECL production.

The addressed limitations of the previously reported work³² include a) the insolubility of the Ru catalyst used in aqueous *tert*-butanol, b) the tendency of phase separation between high-salt (MgCl₂) aqueous solutions and *tert*-butanol leading to heterogeneity, c) the absence of pH control, d) a narrowly explored substrate scope, and e) the formation of significant amounts of side products arising from catalyst decomposition, which limits the yield of the reaction. Furthermore, we demonstrate 1) the applicability of the developed conditions to a single-substrate DECL as a way to test for susceptibility to varying DNA sequences, 2) the maintenance of DNA integrity post-RCM via sequencing analysis, and 3) the first successful stapling of an unprotected on-DNA [i, i+4] peptide as an illustration of the robust functional group tolerance of the developed conditions.

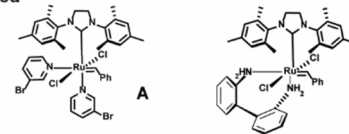
RESULTS AND DISCUSSION

Our work relies on three key findings: 1) a solvent system that allows for homogeneous reaction conditions with much lower catalyst loading, 2) a nonclassical acidic “buffer” that not only avoids basicity but also masks coordinating functional groups—in addition to the effect of MgCl₂, and 3) the use of a decomposition-resistant catalyst, **B**, that minimizes side reactions and maximizes conversion. Our developed reaction conditions are presented in Scheme 1, and our studies toward its development and application are discussed in the following sections.

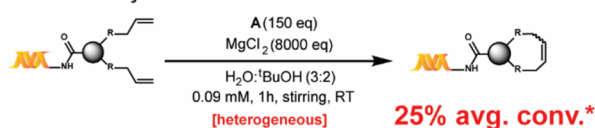
Development of a Solvent System for Reaction Homogeneity. To enhance the reproducibility of RCM on substrates with variably sized DNA tags at a range of scales, our initial work focused on developing a fully homogeneous catalyst–aqueous solvent system combination. Unfortunately, many conventional RCM catalysts (e.g., Grubbs I, Grubbs II, Hoveyda–Grubbs I, Hoveyda–Grubbs II, Grela) are extremely insoluble in most aqueous alcoholic mixtures and were ultimately found to be unproductive for on-DNA RCM under a variety of conditions. Ammonium-functionalized RCM catalysts (e.g., Aquamet)^{30,33} have high solubility in aqueous solutions but were also unproductive for on-DNA RCM, presumably due to DNA–ammonium interactions.³⁴ Even the fast-initiating Grubbs III catalyst **A** (Scheme 1) utilized in the previously reported on-DNA RCM exhibits limited solubility in alcohols (soluble up to 1 mM in pure ^tBuOH). In addition, most nonalcoholic water-miscible solvents (e.g., 1,2-dimethoxyethane or 1,4-dioxane) are metal-coordinating and generally suppress

Scheme 1. Reaction Conditions and Performance Summary of This Work versus Previously Reported Work⁴²

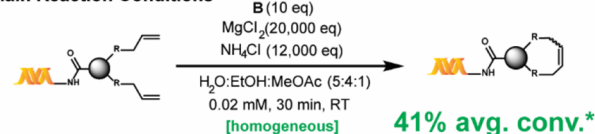
i) Catalysts used



ii) Previous work by Lu et al.



iii) Main Reaction Conditions



^a(i) Grubbs third generation catalyst **A** and its 2,2'-bipyridine derivative **B**; (ii) previously reported conditions for the on-DNA RCM and CM reactions; (iii) our main conditions for the on-DNA RCM and CM reactions; *average percent conversion for the investigated substrate scope (22 substrates).

metathesis. Informed by reports of conventional RCM in ethyl acetate as solvent,³⁵ we were pleased to discover methyl acetate as a much better solvent for both catalysts **A** and **B** (~4 mM). When a methyl acetate/ethanol/water solvent mixture was used, fully homogeneous reaction mixtures were observed, even for solutions at high ionic strengths. Due to the fixed solubility of **B** in methyl acetate, a reaction concentration of only 0.02 mM was possible to maintain adequate solvent percentages. While high dilution favors intramolecular metathesis, it can also stress the catalyst³⁶—a trade-off we attempted to mitigate through the use of the more robust catalyst **B**. Additionally, a reaction concentration of 0.02 mM would be manageable during DECL production, which is the central motivation behind our efforts.

Development of a Brønsted Acidic “Buffer”. To avoid catalyst degradation at basic pH³⁷ and to enhance the masking of coordinating functional groups, we sought to include an acidic buffering system. Despite numerous reports of RCM in the presence of aqueous buffers,^{38,39} we found that even modest amounts of common buffers (e.g., phosphate) or “noncoordinating” buffers^{40,41} (e.g., 2-(*N*-morpholino)ethanesulfonic acid) inhibited reactivity. Hypothesizing the suppression was due to undesired coordination of the buffer’s nonhalide conjugate bases to the Ru center, we considered whether the inclusion of large amounts of NH₄Cl could induce an acidic pH without presenting RCM-suppressing buffer anions. Although not a buffer in the traditional sense, NH₄Cl may block Lewis-base interactions via protonation and/or hydrogen-bonding (e.g., a 4 M aqueous solution of NH₄Cl has a pH of ~5). Indeed, as illustrated in Table 1, adding NH₄Cl significantly enhances conversion (e.g., entries 2 vs 9). With a homogeneous solvent system capable of tolerating high ionic strength in hand, we optimized the proportions of NH₄Cl and MgCl₂ for both high conversion and quality of the LC-MS trace. Ultimately a combination of 20,000 equiv of MgCl₂ and 12,000 equiv of NH₄Cl for 30 min with 10 equiv of catalyst⁴² was found to be optimal (Table 1).

Application of a Decomposition-Resistant Catalyst. Despite these encouraging results, conversions remained limited by the formation of side products. These appeared to originate in

Table 1. Screen for the Optimal Equivalences of MgCl₂ and NH₄Cl^g

Trial# ^a	x	y	% conv. 1b ^b	Trial# ^a	x	y	% conv. 1b ^b
1	0	0	0	9	4000	12000	60 ^d
2	4000	0	13	10	4000	16000	61
3	4000	4000	31	11	20000	12000	72
4	8000	4000	47	12	20000	12000	78 [*]
5	12000	4000	60	13	20000	12000	72 ^Δ
6	16000	4000	69	14	20000	12000	76 [¥]
7	20000	4000	65 ^c	15	20000	0	72 ^e
8	4000	8000	34	16	0	12000	68 ^f

^aAll reactions were run with 1 nmol of **1a**. ^bThe percent conversions (% conv.) were determined by LC/MS after the quenching procedure, as described in the Supporting Information. ^cThe MS trace was of higher quality relative to that of Trial 6 despite a lower conversion. ^dThe MS trace was of higher quality relative to Trial 10. ^eThe MS signals for those were of lower quality relative to Trial 10. ^fCatalyst B instead of A. ^gCatalyst B instead of A, reaction time 5 min. ^hCatalyst B instead of A, reaction time 60 min.

the reduction of one or both olefins, the loss of methylene from one of the olefins and/or the isomerization of the starting material to an unreactive internal olefin (see the Supporting Information). Unfortunately, attempts to suppress those undesired reactions with standard additives such as benzoquinones³⁶ were unsuccessful (various benzoquinones were incompatible with NH₄Cl and also degraded DNA). While several catalytic species have been proposed as culprits behind such side reactions,^{37,43,44} a compelling study by Fogg and co-workers⁴⁵—showing that the addition of substoichiometric amounts of a poisoning ligand limits isomerization—proved to be key to our discovery of the adequacy of **B** for our purposes. Indeed, we found that the addition of 2,2'-biphenyldiamine, **C**, to **A**, as shown in Scheme 2(ii), enhances the percent conversion

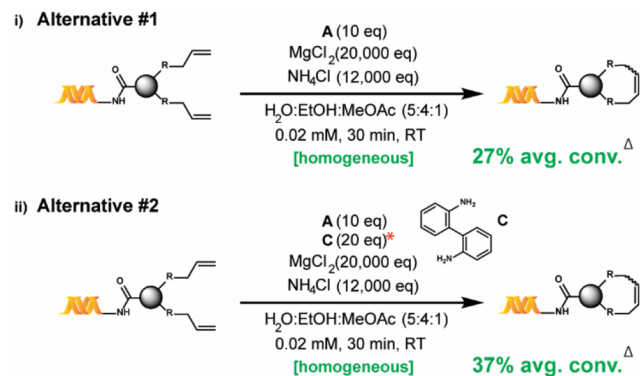
in situ formation of **B** was responsible for the conversion enhancement observed. The substitution of **B** for **A**, as shown in Scheme 1(iii), indeed proved to significantly enhance conversion beyond what an **A**–**C** combination could achieve (Tables 2–4). Since catalyst **B** was not commercially available at the time of the study and given its reported synthesis requires a glovebox,⁴⁶ we also studied alternative reaction conditions involving catalyst **A** instead (Scheme 2).

Of note, reactions within DECLs are typically performed on nano- to micromole scales, and mass spectrometry is the main method of characterization. Therefore, to further corroborate the formation of the putative cyclized product, we developed a chemical derivatization protocol through OsO₄-mediated dihydroxylation, which reveals the number of olefins present (Scheme 3). Results of the dihydroxylation of the post-RCM reaction mixture of **1a** were consistent with the formation of the cyclized product as the major product (see the Supporting Information).

We next turned to applying these four conditions—the previously reported conditions from Lu et al. (Scheme 1, (ii)), our main reaction conditions (Scheme 1, (iii)), and the two alternative conditions (Scheme 2)—to a series of substrate scope studies (Tables 2–4). Overall, while the conditions reported by Lu et al. exhibit comparable conversions for simple substrates, significant differences were observed for those containing unfavorable coordinating functional groups.

We first investigated the ring-size scope of the reaction (Table 2). Although all four conditions generally produced similar results, the ^tBuOH/MgCl₂ system significantly underperformed in the case of **3a**. This may stem from the presence of the glycol chain within this substrate. Of note, these reactions were complete after only 30 min at room temperature, which is remarkable given it can take days and high temperatures for rings of comparable size to close in organic media.²⁴

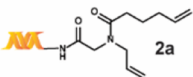
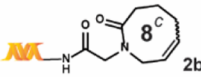
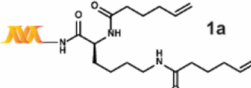
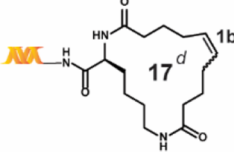
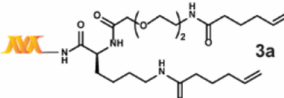
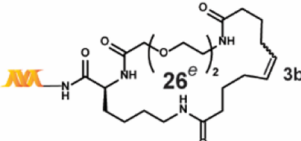
To investigate the functional group tolerance of the four reaction conditions, we synthesized a series of substrates differentiated only by substitution at a single position (Table 3). Using substrate **4a** (R = H) as benchmark, we were able to demonstrate

Scheme 2. Alternative Reaction Conditions using Catalyst A^a

^aThe screening results used to determine the optimal equivalence of **C** are provided in the Supporting Information, Table S1. ^Δ Average percent conversion for the investigated substrate scope (22 substrates).

(see the Supporting Information). However, we were aware of another study by Fogg and co-workers⁴⁶ which reported the 40% equilibrium yield of decomposition-resistant catalyst **B** from **A** in the presence of **C**, at RT. We therefore suspected that the

Table 2. Ring Size Scope Study

Diene ^a	Desired Product	% conv. ^b
 2a	 2b	62 (73) (33) (65)
 1a	 1b	78 (75) (72) (70)
 3a	 3b	70 (61) (58) (23)

^aAll reactions were run with 1 nmol of each diene. ^bThe percent conversions (% conv.) were determined by LC/MS after quenching, as described in the Supporting Information. ^{c-e}Ring sizes in terms of number of member atoms; reaction conditions: green = main reaction conditions, blue = alternative #2, black = alternative #1, and red = Lu et al.

Scheme 3. Confirmation of Ring Formation via Chemical Modification

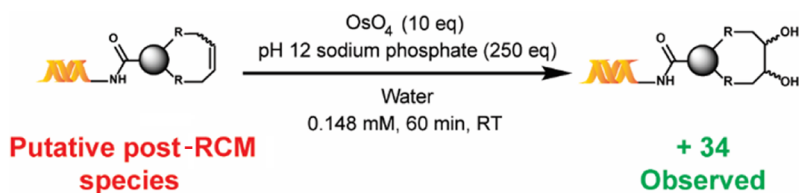
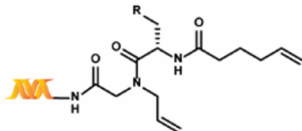
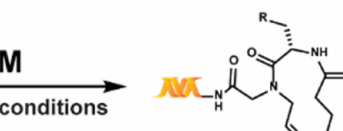
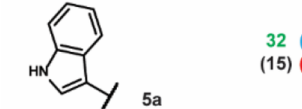



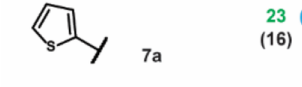





Table 3. Functional Group Tolerance Study

R ^a	% conv. ^b	R ^a	% conv. ^b
 4a	48 (39) (35) (16)	 9a	23 (11) (nd) (nd)
 5a	32 (15) (15) (<5)	 10a	35 (15) (11) (5)
 6a	59 (54) (38) (30)	 11a	55 (35) (36) (20)
 7a	23 (27) (16) (9)	 12a	46 (28) (20) (21)
 8a	28 (28) (25) (<5)	 13a	48 (33) (36) (22)

^{a,b}As in Table 2; reaction conditions: green = main reaction conditions, blue = alternative #2, black = alternative #1, and red = Lu et al.; nd = not determined. Note that no thiol-containing substrate was included due to dimerization via disulfide formation between DNA conjugates.

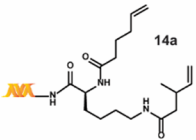
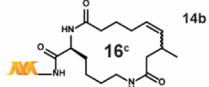
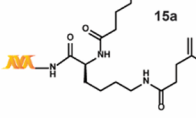
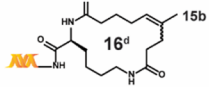
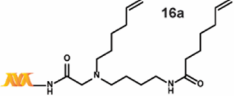
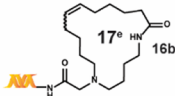
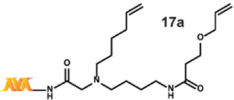
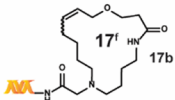
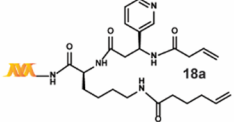
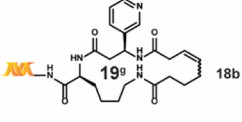
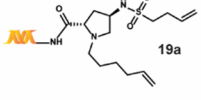
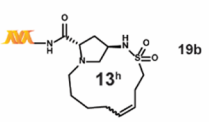
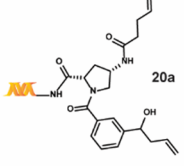
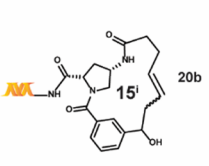
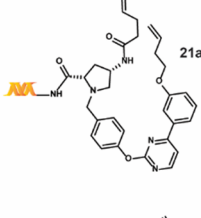
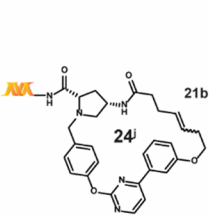
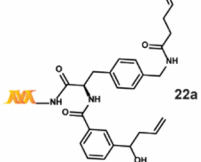
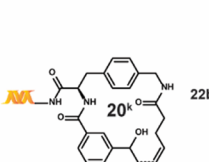
the remarkable functional group tolerance of our main reaction conditions. Indeed, functional groups such as the carboxylic acid of **8a** or the amine of **13a**—groups typically protected in organic-phase RCM—exhibited at least half the conversion obtained for reference compound **4a**. This suggests that the incorporation of NH_4Cl exerts an acidic masking effect, consistent with other reports of RCM in the presence of acidic additives.^{47,48} In contrast, the previously reported unbuffered $\text{MgCl}_2/\text{tBuOH}$ condition exhibited significant sensitivity to the indole of **5a**, the thiophene of **7a**, the carboxylic acid of **8a**, and the phenol of **10a**.

We also tested a series of on-DNA compounds designed to investigate other DECL-relevant substrate features (Table 4). While we had previously observed that internal olefins were unreactive to our RCM conditions, we sought to assess the impact of small substituents in β -methyl **14a** and α -methyl **15a**. When compared to the analogous unsubstituted **1a**, **14a** and **15a** were cyclized in modest conversions, suggesting a low-tolerance for steric-encumbrance at or near the olefin. The presence of an allylic chalcogen is known to enhance RCM efficiency through a preassociation effect.⁴⁹ However, comparable conversions were observed for alkyl **16a** and related allyl ether **17a**. The proximity of coordinating groups to the olefin may also inhibit successful cyclization. Although all four RCM conditions effected negligible cyclization on **18a** and **19a**—bearing a proximally located pyridyl and sulfonamide, respectively—a modest amount of cyclization was observed for **21a**—exhibiting a more distally located pyrimidine. Homoallylic alcohols are a potential substrate series for on-DNA RCM, as the water-compatible allylation of aldehydes is well-known.⁵⁰ Homoallyl alcohols **20a** and **22a** were successfully cyclized, although sterically encumbered **20a** afforded lower conversion. Finally we applied these conditions to the CM of 5-hexenoic acid with the CM-favored thioether substrate⁵¹ **23a**. All four conditions provided the CM product in moderate to good conversion (Scheme 4).

Certain on-DNA reactions exhibit sensitivity to the length of the DNA-tag, which may be due to differences in solubility and/or intermolecular DNA interactions. To test the viability of our new RCM conditions on a late-stage DECL substrate, we prepared 56-bp dsDNA substrate **24a** from 17-bp dsDNA substrate **1a**. Application of our main reaction conditions (Scheme 1, iii) to **24a** afforded **24b** at 69% conversion, which was close to the 78% conversion observed for **1a** (Scheme 5). After an adapted precipitation procedure to minimize chaotropic effects (see the Supporting Information), the 56-bp dsDNA was obtained in 20% yield (quantified by Bioanalyzer electropherogram analysis). Although the 56-bp dsDNA was the dominant component within the post-RCM reaction mixture, small amounts of larger DNA segments were observed (see the Supporting Information), which may arise from intermolecular metathesis. These may require removal by HPLC during full-scale DECL synthesis.

The application of newly developed on-DNA conditions to a pilot DECL and subsequent analysis by DNA sequencing are useful tests prior to beginning a full DECL production. To investigate the potential for DNA base-related effects, we prepared a small single-substrate DECL of ~47,000 unique 56-bp dsDNA sequences from substrate **1a**. This DECL was built through a three-cycle “split-and-pool” approach, featuring three cycles of splitting into portions, performing 36 ligations of unique dsDNA tags, and pooling. All DNA tags used within a cycle were of identical molecular weight, and thus the DECL was observed as a single ensemble mass after purification by HPLC (see the Supporting Information). The application of our main reaction conditions to the DECL resulted in the observable loss of

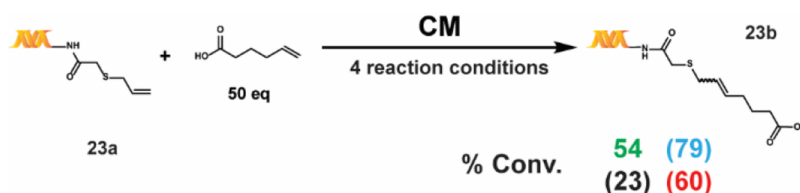
Table 4. Additional Substrate Scope Study

Diene ^a	Desired Product	% conv. ^b
		16 (15) (10) (13)
		8 (7) (7) (5)
		46 (45) (40) (26)
		49 (58) (63) (29)
		<5 (6) (<5) (<5)
		<5 (<5) (<5) (<5)
		16 (18) (7) (23)
		33 (34) (22) (32)
		90 (100) (67) (88)

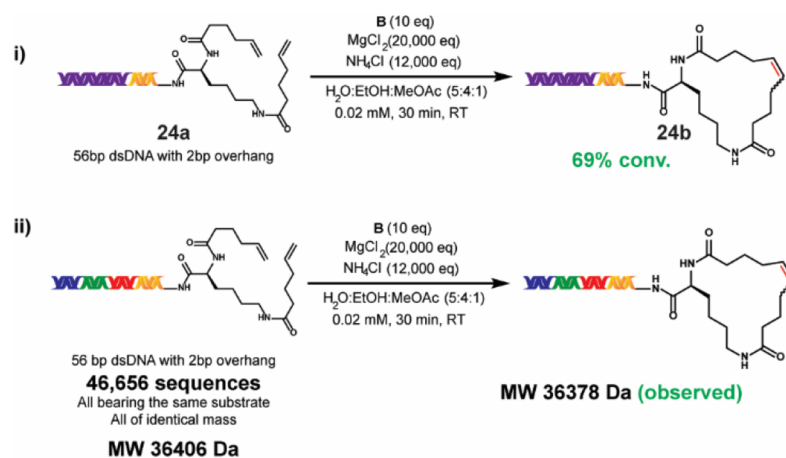
^{a,b}As in Table 2. ^{c-k}Ring sizes in terms of number of member atoms; reaction conditions: green = main reaction conditions, blue = alternative #2, black = alternative #1, and red = Lu et al.

MW = 28 (loss of ethylene from RCM cyclization). Additionally, sequenced samples of the DECL before and after the RCM reaction showed no significant differences, thus ascertaining DNA integrity post-RCM (see the Supporting Information).

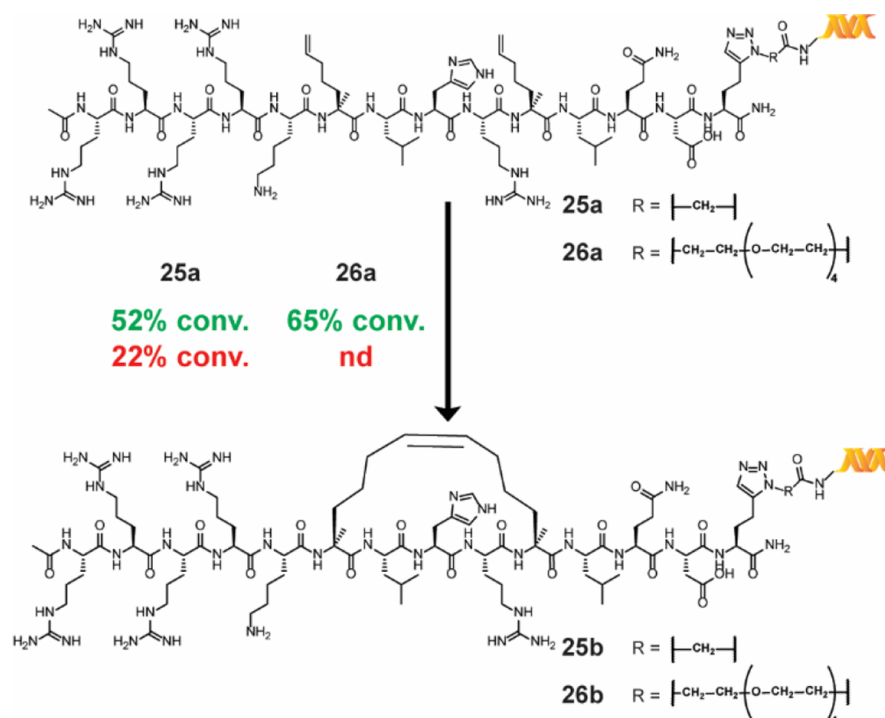
In addition to the synthesis of small-molecule macrocycles, RCM has also been used to prepare stapled peptides—linear peptides conformationally constrained through an intramolecular

Scheme 4. On-DNA Cross Metathesis (CM)^a

^aThe reaction was run with 1 nmol of 23a. A 50 mM stock solution of the 5-hexenoic acid building block was prepared in ethanol. The percent conversions (% conv.) were determined by LC/MS after the quenching procedure, as described in the [Supporting Information](#). Reaction conditions: green = main reaction conditions, blue = alternative #2, black = alternative #1, and red = Lu et al.

Scheme 5. Application of the Main Reaction Conditions to a 56-bp DNA Substrate and to a Single-Substrate DECL^a

^a(i)The reaction was run with 5 nmol of 24a. (ii) The reaction was run with 5 nmol of the single-substrate DECL.

Scheme 6. RCM Stapling of an Unprotected [i, i+4] Peptide Connected to DNA via Two Different Linkers^a

^aEach reaction was run with 1 nmol of material. The percent conversion (% conv.) was determined by LC/MS after the quenching procedure, as described in the [Supporting Information](#). Reaction conditions: green = main reaction conditions, and red = Lu et al.

linkage—to simulate protein–protein interactions.⁵² To test the relevance of our reaction conditions for the production of

on-DNA stapled peptides, we synthesized substrates 25a and 26a through copper-catalyzed azide–alkyne cycloaddition of an

alkynylated [i, i+4] SRC stapled peptide precursor known to bind the coactivator region of ER α .⁵³ The application of our main reaction conditions to both substrates provided the stapled peptide in moderate conversions while, in the case of **25a**, significantly outperforming the conditions reported by Lu et al. (Scheme 6)—the comparison was not studied for **26a**. To further corroborate the successful production of the stapled peptide, adapted samples of a control, peptide **26a**, and stapled peptide **26b** were tested within a homogeneous time-resolved fluorescence (HTRF) assay for binding to the coactivator region of ER α . Both **26a** and **26b** displayed a dose-dependent HTRF response, and significantly enhanced coactivator region binding was observed for stapled peptide **26b** (see the [Supporting Information](#)). It is noteworthy that DECL technology is analogous to phage display libraries.⁵⁴ However, our methodology is particularly useful for incorporating unnatural amino acids such as the α -methyl- α -pentenyl amino acid required for RCM stapling. Although highly specialized systems for incorporating unnatural amino acids for phage display have been reported,⁵⁵ there have been no reports of making stapled peptides by phage display.

CONCLUSIONS

In summary, we have developed a RCM reaction system promoting homogeneity, minimization of side reactions, and masking of coordinating functional groups and 1) studied and contrasted its applicability to a diverse range of substrates, 2) ensured its compatibility to DNA through the successful cyclization of a single-substrate DECL, and 3) demonstrated its functional group tolerance through the production of the first on-DNA stapled peptide. We believe this work will allow for the integration of the well-documented capacity of RCM to generate chemical diversity into DECLs. Efforts to apply this work toward the production of RCM-based DECLs and their screening are ongoing within these laboratories and will be reported in due course.

EXPERIMENTAL PROCEDURES

Each RCM substrate was constructed from a 17-bp dsDNA DNA headpiece (see the [Supporting Information](#) for structure) and was purified by HPLC due to RCM-inhibiting effects induced by residual chemical building block impurities. Procedural details for all four RCM methods are included in the [Supporting Information](#). Additionally, while reaction mixtures involving 17-bp dsDNA tagged substrates were fully homogeneous, those with longer dsDNA tags (56 bp dsDNA) tended to be slightly cloudy. While this did not appear to affect the reaction conversion, a specific precipitation procedure was required to obtain suitable DNA recovery (see the [Supporting Information](#)). For application to substrates with DNA tags of alternative sizes (>56-bp) or composition, adjustments may be required to ensure substrate solubility.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscmbosci.9b00199>.

General procedures and reaction conditions; catalyst and substrate synthesis information; RCM-specific experimental procedures; experiments for confirmation of RCM cyclization; DNA sequencing results for verification of DNA integrity post-RCM; characterization information; postmetathesis MS deconvolution spectra ([PDF](#))

AUTHOR INFORMATION

Corresponding Authors

Damian W. Young – Baylor College of Medicine, Houston, Texas; orcid.org/0000-0002-1595-9658;

Email: Damian.young@bcm.edu

Nicholas Simmons – Baylor College of Medicine, Houston, Texas; orcid.org/0000-0003-0766-5382;

Email: nsimmons@its.jnj.com

Other Authors

Olivier B. C. Monty – Baylor College of Medicine, Houston, Texas, and Rice University, Houston, Texas

Pranavanand Nyshadham – Baylor College of Medicine, Houston, Texas

Kurt M. Bohren – Baylor College of Medicine, Houston, Texas

Murugesan Palaniappan – Baylor College of Medicine, Houston, Texas

Martin M. Matzuk – Baylor College of Medicine, Houston, Texas

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acscmbosci.9b00199>

Author Contributions

[§]O.B.C.M. and N.S. contributed equally. 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

CM, cross metathesis; DECL, DNA-encoded chemical library; DNA, DNA; ER α , estrogen receptor alpha; HPLC, high performance liquid chromatography; HTRF, homogeneous time-resolved fluorescence; RCM, ring-closing metathesis

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