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

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ABSTRACT: Reaction heterogeneity, poor pH control, and catalyst decomposition in the ring-closing metathesis (RCM) of DNA–chemical conjugates leads 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.

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 oligomers[3] and in high-throughput DNA-sequencing methods[4] 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 use,[6] and, most importantly, offer the opportunity for deeper exploration of chemical space[5] 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 is 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 have 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 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) reaction[29] has been demonstrated in aqueous *tert*-butanol, using a high excess (10,000 eq) of $MgCl_2$ 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 $MgCl_2$ (400 eq).[30] The Mg^{2+} 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 3rd generation Grubbs catalyst/$MgCl_2$/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.

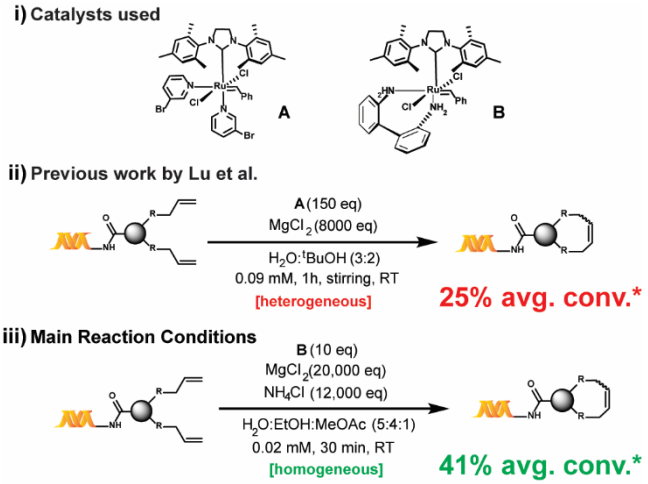
The addressed limitations of the previously reported work[32] include a) the insolubility of the Ru catalyst used in aqueous *tert*-butanol, b) the tendency of phase separation between high-salt ($MgCl_2$) aqueous solutions and *tert*-butanol leading to heterogeneity, c) the absence of pH control, d) 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 non-classical acidic "buffer" that not only avoids basicity but also masks coordinating functional groups—in addition to the effect of $MgCl_2$, 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 towards its development and application are discussed in the following sections.

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



i) Grubbs 3rd generation catalyst **A** and its 2,2'-biphenyldiamine 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).

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 ^{t}BuOH). In addition, most non-alcoholic water-miscible solvents (e.g., 1,2-dimethoxyethane or 1,4-dioxane) are metal-coordinating and generally suppress 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 catalyst[36]—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 pH[37] 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 "non-coordinating" buffers[40,41] (e.g., 2-(N-morpholino)ethanesulfonic acid) inhibited reactivity. Hypothesizing the suppression was due to undesired coordination of the buffer's non-halide conjugate bases to the Ru center, we considered whether the inclusion of large amounts of NH_4Cl could induce an acidic pH without presenting RCM-suppressing buffer anions. Although not a buffer in the traditional sense, NH_4Cl may block Lewis-base interactions via protonation and/or hydrogen-bonding (e.g., a 4M aqueous solution of NH_4Cl has a pH of ~5). Indeed, as illustrated in Table 1, adding NH_4Cl significantly enhances conversion (e.g. entries 2 vs 9). With a homogenous solvent system capable of tolerating high ionic strength in hand, we optimized the proportions of NH_4Cl and $MgCl_2$ for both high conversion and quality of the LC-MS trace. Ultimately a combination of 20,000 equiv. of $MgCl_2$ and 12,000 equiv. of NH_4Cl for 30 min with 10 equiv. of catalyst[42] was found to be optimal (Table 1).

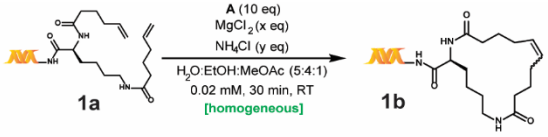
Application of a Decomposition-Resistant Catalyst.

Despite these encouraging results, conversions remained limited by the formation of side products. These appeared to originate in 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 Supporting Information). Unfortunately, attempts to suppress those undesired reactions with standard additives such as benzoquinones[36] were unsuccessful (various benzoquinones were incompatible with NH_4Cl 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 coworkers[45]—showing that the addition of sub-stoichiometric 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 (see Supporting Information). However, we were aware of another study by Fogg and coworkers[46] 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 *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 glove box,[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 OsO_4-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 Supporting Information).

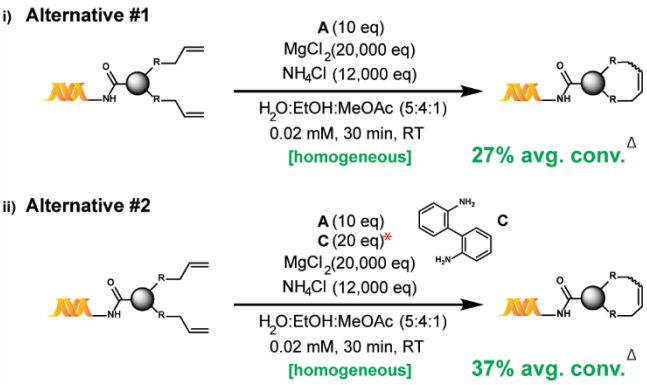
Table 1 Screen for the Optimal Equivalences of $MgCl_2$ and NH_4Cl



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]

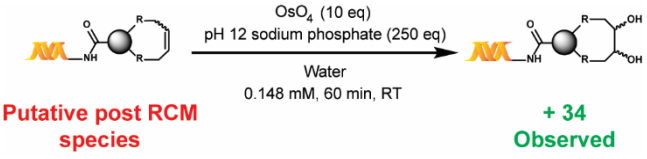
[a]All reactions were run with 1 nmol of **1a**. [b] The percent conversions (% conv.) were determined by LC/MS after the quenching procedure, as described in the Supporting Information. [c] The MS trace was of higher quality relative to that of Trial 6 despite a lower conversion. [d] The MS trace was of higher quality relative to Trial 10; * Catalyst **B** instead of **A**; Δ Catalyst **B** instead of **A**, reaction time 5 min; ¥ Catalyst **B** instead of **A**, reaction time 60 min; [e, f] The MS signals for those were of lower quality relative to Trial 10.

Scheme 2. Alternative Reaction Conditions using Catalyst A



*The 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).

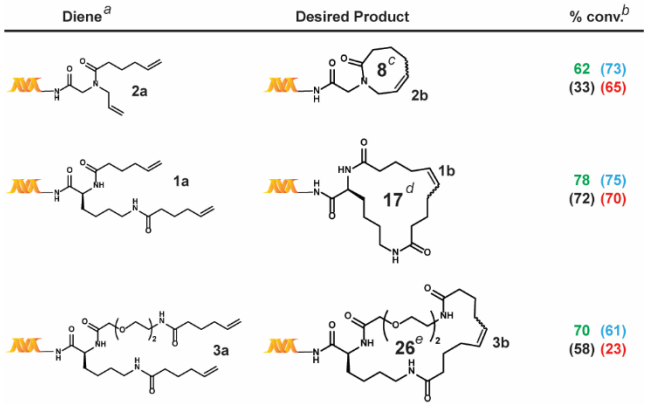
Scheme 3. Confirmation of Ring Formation via Chemical Modification.



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 ^{t}BuOH/$MgCl_2$ 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 minutes 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]

Table 2. Ring Size Scope Study

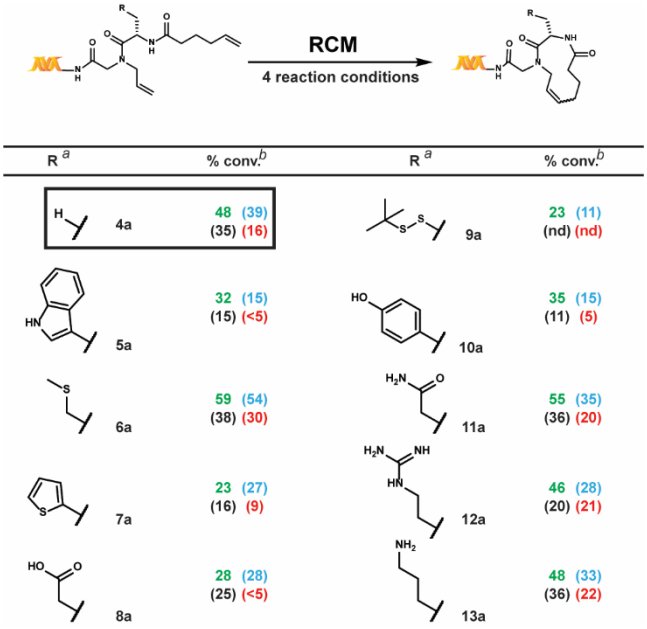


[a] All reactions were run with 1 nmol of each diene; [b] The percent conversions (% conv.) were determined by LC/MS after quenching, as described in the Supporting Information; [c-e] Ring sizes in terms of number of member atoms; Reaction conditions: Green = Main Reaction Conditions, Blue = Alternative #2, Black = Alternative #1 and Red = Lu et al.

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 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 $MgCl_2$/^{t}BuOH 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

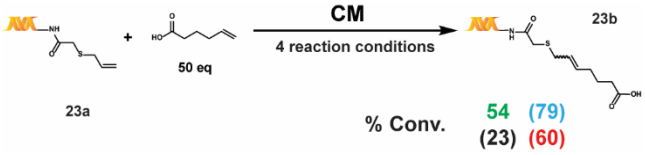
Table 3. Functional Group Tolerance Study



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.

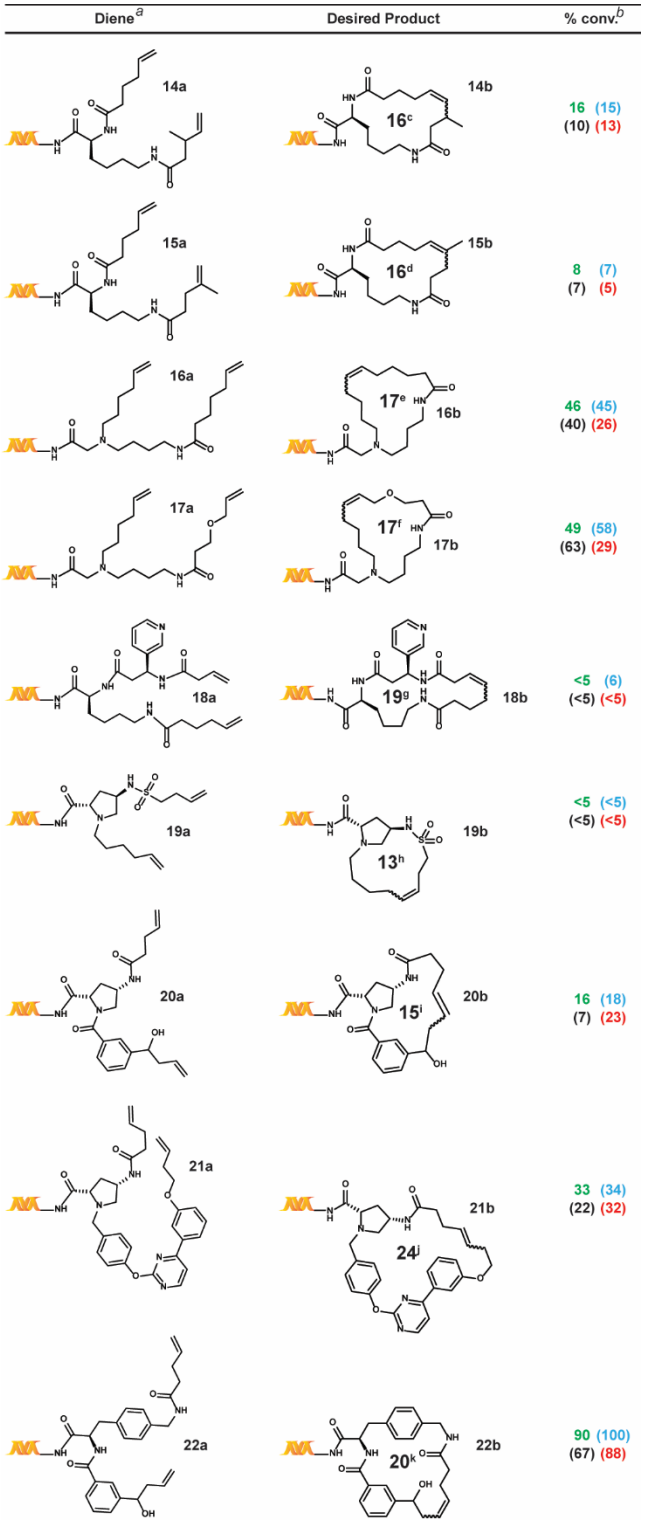
steric-encumbrance at or near the olefin. The presence of an allylic chalcogen is known to enhance RCM efficiency through a pre-association effect.[49] However, comparable conversions were observed for alkyl **16a** and related allyl ether **17a**. The proximity of coordinating groups to the olefin may also inhibit successful cyclization. Although all four RCM conditions effected negligible cyclization on **18a** and **19a**—bearing a proximally-located pyridyl and sulphonamide, 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 substrate[51] **23a.** All four conditions provided the CM product in moderate to good conversion (Scheme 4).

Scheme 4. On-DNA Cross Metathesis (CM)



The 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.

Table 4. Additional Substrate Scope Study

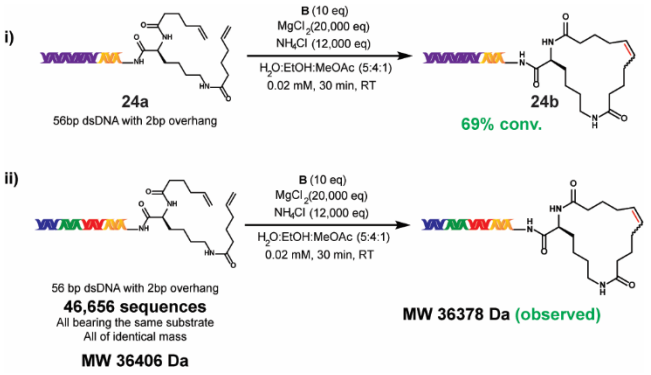


a, b As in Table 2; c-k Ring sizes in terms of number of member atoms; Reaction conditions: Green = Main Reaction Conditions, Blue = Alternative #2, Black = Alternative #1 and Red = Lu et al.

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 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 Supporting Information), which may arise from intermolecular metathesis. These may require removal by HPLC during full-scale DECL synthesis.

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

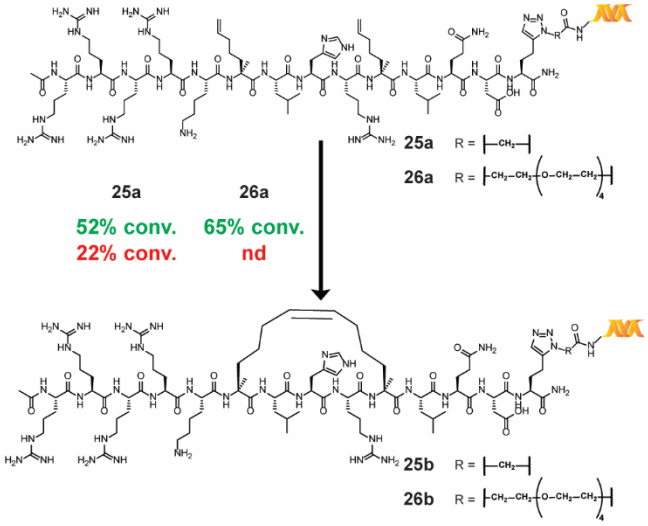


i) The reaction was run with 5 nmol of **24a**; ii) The reaction was run with 5 nmol of the single-substrate DECL.

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 Supporting Information). The application of our main reaction conditions to the DECL resulted in the observable loss of 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 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 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 ERα.[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 ERα. Both **26a** and **26b** displayed a dose-dependent HTRF response and significantly enhanced co-activator region binding was observed for stapled peptide **26b** (see 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.

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



Each 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.

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 towards 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 17-bp dsDNA DNA headpiece (see 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 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.
Content: general procedures and reaction conditions; catalyst and substrate synthesis information; RCM-specific experimental procedures; experiments for the confirmation of RCM cyclization; DNA sequencing results for verification of DNA integrity post-RCM; characterization information; post-metathesis MS deconvolution spectra.
This material is available free of charge via the Internet at http://pubs.acs.org."

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / §These authors contributed equally.

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ABBREVIATIONS

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

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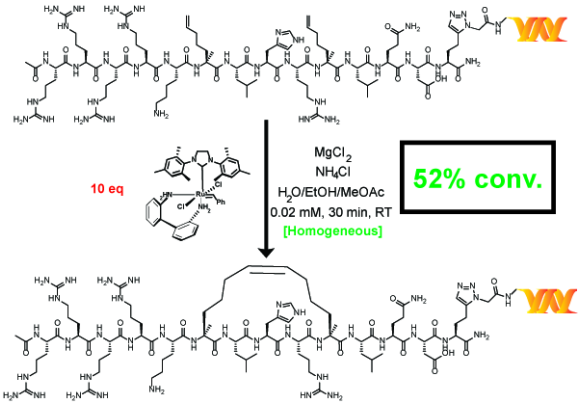

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