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Constructive on-DNA Thiol Aerial Oxidization for DNA-Encoded Library Synthesis

Lijun Xue, Hangke Ma, Weijie Liu, Nicolas Duchemin, Fanming Zeng, Jie Zhang, Huanqing Zhang, Kexin Yang, and Yun Jin Hu*

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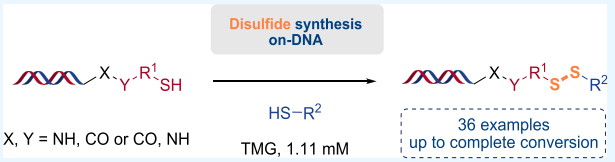
ABSTRACT: A novel on-DNA oxidative disulfide formation method has been developed. Under ambient conditions, the methodology showcased wide applicability and swift implementation in routine DNA-encoded library synthesis to access pharmaceutically relevant motifs.



Despite its relatively recent inception,[1,2] DNA-encoded library technology (DELT) has been broadly recognized as a promising platform for hit identification in drug discovery.[3] Creating broad chemical space from massive numbers of on-DNA compounds,[4] necessitating a particularly simple and efficient affinity selection method,[5,6] and benefiting from well-established algorithm-driven data analysis processes to discriminate binding DEL members from the nonbinders,[7] the technology has raised the interest of both academia and industry and is now regarded as one of the most strategic pathways to generate rapidly relevant chemical matter. The significance and strong impact of DELT in unveiling relevant hits in an efficient and economical manner was recently witnessed by the expedient access to SARS-CoV-2 M^{pro} inhibitors[8] and naturally became increasingly significant in drug discovery.[9,10] This effort notably contributed to the discovery of phase 2 clinical candidates, sEH inhibitor GSK2256294 and RIP1 inhibitor GSK2982772 from GSK and recently FDA-approved, and autotaxin inhibitor X-165 for phase 1 clinical trials.[9,11,12]

Nonetheless, while the field has been thriving, there is still a confined panel of DEL-applicable chemical transformation that one can routinely use when designing and synthesizing DELs. The fragile nature of oligonucleotides, the prerequisite presence of an aqueous medium, and the wide substrate applicability necessary to any methodology used in combinatorial synthesis are natural hurdles to the development of innovative on-DNA chemistry, would it be inspired by off-DNA organic synthesis or not. Hence, many efforts to broaden the scope of the DNA-compatible chemical reaction toolbox, to apply these methods for DEL synthesis, and to expand the chemical diversity of high-quality libraries are endeavored by many groups,[13−18] including ours.[19−24] Motivated by the expansion of DEL-available chemical space, we set our minds on the development of the synthesis of a naturally predominant motif: the disulfide bond.

Organic compounds containing disulfides play crucial roles in chemistry and biology[25,26] as they are found in many extracellular peptides and proteins and are a true prerequisite for their biological activity.[27] They bring conformational support and increase their stability. Disulfide bonds are also a very common motif in animal and plant peptide toxins.[28] These disulfide-rich peptides typically bind very selectively and with high affinities to their targets. As potential therapeutics, disulfide-containing compounds are agents used in numerous therapeutic applications: antiradiation,[29] antiplatelet aggregation,[30] and antithrombotic,[31] antiatherogenic,[32] antileishmanial,[33] antioxidant and anti-inflammatory,[34] and other therapeutic uses.[35]

Hence, disulfide compounds have been increasingly considered attractive organic molecules for drug discovery, and several highly relevant molecules illustrate their pharmaceutical amenability (Figure 1). Oxiglutatione, a disulfide peptide dimer, is prescribed for breast cancer therapy and the treatment for chronic obstructive pulmonary diseases.[36] Fursultiamine is used for vitamin-related disorders,[37] while lipoic acid is employed for the treatment of hearing loss, diabetic neuropathy, multiple sclerosis, and biocefalin cognition disorders.[38] Biocefalin is used to increase the cerebral brain activity in patients with impaired cerebral function,[39] and Pantethine, anticataract agents, is prescribed for the treatment of lipoprotein and vitamin-related disorders.[40]

Witt recently conducted an in-depth review of the synthesis of disulfides and outlined the most common methods for gaining access to this motif.[41] The most common method for

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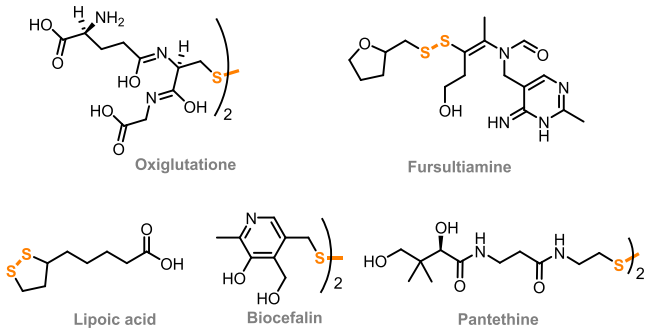


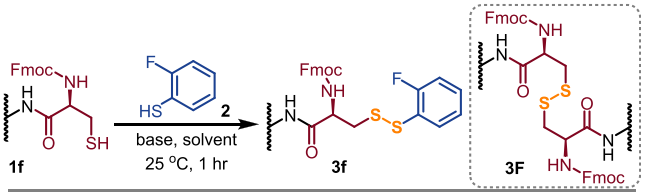
Figure 1. Known drug molecules with a disulfide motif.

the preparation of both symmetric and unsymmetric disulfides is the oxidation of appropriate thiols. Nonetheless, the efficacy of such methods usually decreases from aromatic thiols through primary and secondary to tertiary alkyl thiols. However, oxidation is mostly served for the synthesis of symmetric disulfides as the implementation of such strategy for unsymmetrical compounds naturally results in a mixture of all possible disulfides. Transition metal-promoted aerial oxidation,[42] iodine or bromine,[43] hydrogen peroxide,[44] DDQ,[45] chromates,[46] and nitrogen-containing oxidants[47] are commonly practiced. Interestingly, symmetrical disulfides can also be obtained by the reductive coupling of sulfonyl chlorides.[48] Also, sulfur monochloride (S_2Cl_2) has been used to prepare disulfides from activated aromatic compounds, alkynes, and alkenes in very good yields.[49]

Additionally, the most widely used method of disulfide formation, besides oxidation of thiols, involves the reaction of sulfenyl derivatives with thiols or thiolate anions by nucleophilic SN_2-S displacement of a suitable leaving group from the sulfenyl precusor.[50] Additionally, the asymmetric disulfide can be made from thiosulfonates, thiosulfates, or thioester.[51] The disulfide to another disulfide exchange reaction can lead to asymmetrical disulfides. The evaluation of the wide variety of reported methods for the synthesis of both symmetric and asymmetric disulfides led us to the conclusion that most of these are hardly translatable to on-DNA chemistry due to restrictions to organic solvents, involving modest to strong oxidant, transitional metal catalyst, strong acidic, or basic conditions. Yet, we hypothesized that the use of aerial oxidization processes could be the most direct and rational approach to access on-DNA disulfide formation. Hence, we turned our focus on such pathways despite known challenges, in hopes that a judicious selection of reaction conditions could overcome such obstacles.

We naturally kicked off our study by a careful screening of the reaction conditions. The DNA conjugate starting material **1f** was prepared from a DNA "headpiece" coupled with a symmetric disulfide and then reduced using dithiothreitol (DTT) to release the corresponding free thiol as a model substrate for reaction condition study (see Supporting Information for details). Such an approach can easily be implemented if the free thiol is not commercially available. 2-fluorothiophenol **2** was used as the model coupling partner. To assess the optimal conditions to achieve the desired on-DNA disulfide formation, numerous factors were evaluated to determine the optimal buffer such as base, reactant usage, temperature, and reaction time (Scheme 1). Initially, the emphasis was placed on the impact of the base nature and quantity.

Scheme 1. Disulfide Formation Condition Optimization



Entry	Base	Base (mM)[b]	2 (mM)[b]	Solvent	3f (%)[c]	3f' (%)[c]
1	/	/	11.11	THF : H_2O = 1 : 2	10	90
2	K_2CO_3	0.56	11.11	THF : H_2O = 1 : 2	0	99
3	NaOH	0.56	11.11	THF : H_2O = 1 : 2	0	80
4	borate buffer (pH 9.5)	0.56	11.11	THF : H_2O = 1 : 2	10	86
5	TMG	0.56	11.11	THF : H_2O = 1 : 2	49	50
6	pyridine	0.56	11.11	THF : H_2O = 1 : 2	32	25
7	TEA	0.56	11.11	THF : H_2O = 1 : 2	45	53
8	TMG	0.56	5.56	THF : H_2O = 1 : 2	29	65
9	TMG	0.56	22.22	THF : H_2O = 1 : 2	66	30
10	TMG	1.11	22.22	THF : H_2O = 1 : 2	79	20
11	TMG	2.22	22.22	THF : H_2O = 1 : 2	79	20
12	TMG	1.11	22.22	MeOH : H_2O = 1 : 2	0	69
13	TMG	1.11	22.22	EtOH : H_2O = 1 : 2	64	28
14	TMG	1.11	22.22	ACN : H_2O = 1 : 2	0	64
15	TMG	1.11	22.22	DMSO : H_2O = 1 : 2	70	7
16	TMG	1.11	22.22	THF : H_2O = 1 : 1	96	2
17	TMG	1.11	22.22	THF : H_2O = 1 : 3	63	36
18	TMG	1.11	22.22	THF : H_2O = 1 : 4	50	50

[a]**1f** (5.0 nmol in H_2O, final concentration was 0.06 mM, 5.0 μL), **2**, base, solvent, V_{total} = 90 μL, 25 °C, 1 h. [b]Final concentration. [c]Conversions based on **1f**. Conversions were determined by LC−MS.

The oxidative disulfide formation was carried out with routinely employed DEL-compatible bases including potassium carbonate, sodium hydroxide, tetramethylguanidine (TMG), borate buffer (pH 9.5), pyridine, triethyl amine, and water as a control conditions (entry 1 to 7). TMG rapidly stood out as the most efficient as an encouraging 49% conversion to **3f** was achieved, while the dimer byproduct **3F** formation was suppressed. The concentration of free thiol **2** was then assessed, and the conversion dramatically improved using 22.22 mM of the thiol partner (entries 8, 9).

Further improvements were achieved with the change of quantity of TMG (entries 9 to 11). Solvent's composition was then scrutinized (entries 11 to 18) and revealed that the reaction afforded the highest conversion when performed in 50% aqueous THF media. Eventually, transformation worked the best under the conditions of 1.11 mM of TMG and 22.22 mM of **2** in 50% aqueous THF at room temperature for 1 h (entry 16).

With the optimum conditions in hand, the substrate scope was explored by first varying the nature of the DNA-conjugate (Scheme 2). Both **3a** with an amide or **3b** with a reverse amide linkage to DNA oligo were well tolerated as well as the extension of linker's length, up to a certain degree (**3d** vs **3b**, **3c**). To our delight, the reaction of alkyl thiol was permitted (**3e** to **3i**) even in the presence of bulky groups (**3f**, 96% conversion). The tolerance to the secondary alkyl thiol showcased that the steric effect does not obstruct the conversion for alkyl substrates (**3g** to **3i**). As well, phenylmethanethiols (**3j**, 78%; **3k**, 94%) and 3 or 4-mercaptophenyl

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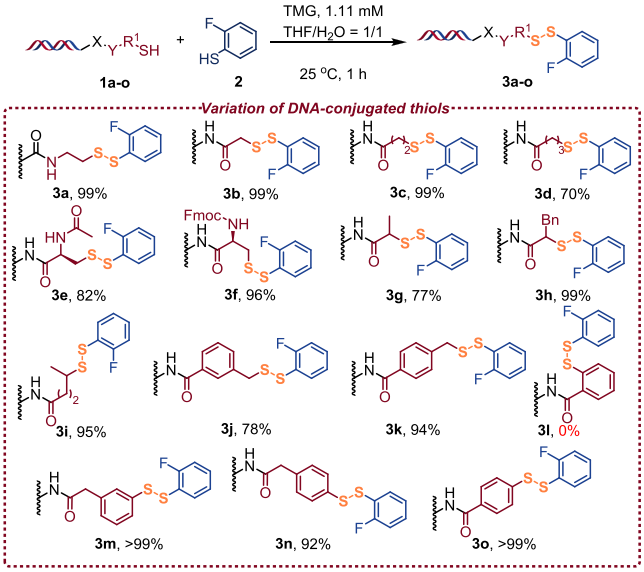
Scheme 2. Substrate Scope of Various on-DNA Thiol Conjugates



[a]**1** (5.0 nmol in H_2O, final concentration was 0.06 mM, 5.0 μL), **2** (100.0 mM in THF, final concentration was 22.22 mM, 20.0 μL), TMG (4.0 mM in THF, final concentration was 1.11 mM, 25.0 μL), V_{H2O} = 40 μL, THF/H_2O = 1/1, V_{total} = 90 μL, 25 °C, 1 h. [b]Conversions based on **1**. [c]Conversions were determined by LC−MS.

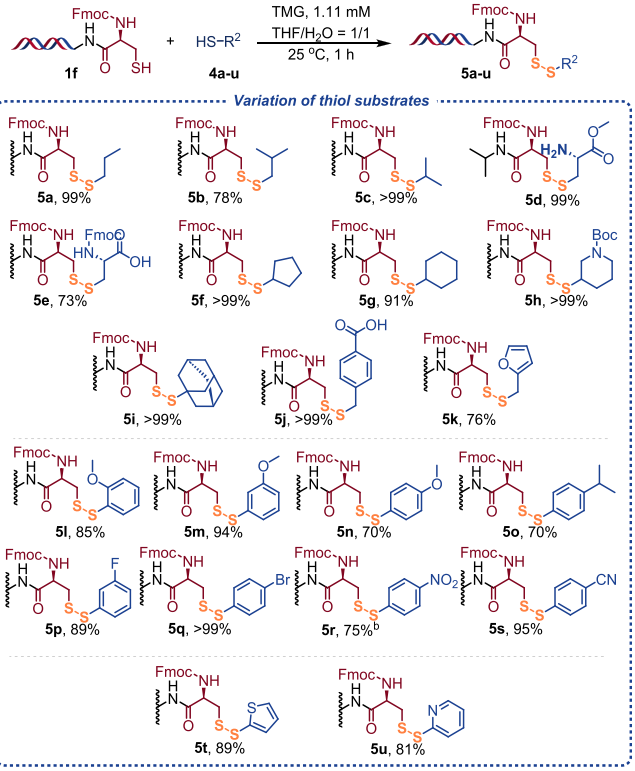
substrate worked well, while 2-mercaptophenyl substrate failed likely due this time to the steric hindrance (**3l** vs **3m**, **3n**, **3o**).

Next, the substrate scope of the off-DNA thiols was explored (Scheme 3). Regardless of the degree of steric hindrance, the conversion of aliphatic acyclic substrates was acceptable to excellent (**5a** to **5e**). Interestingly, thiols with free amine were also reacted smoothly (**5d**).

We naturally moved to the evaluation of cycle-containing compounds and cyclic aliphatic thiol substrates were well permitted for the disulfide formation (**5f** to **5i**), including particularly bulky substrate (**5i**, >99%) as well as 4-(mercaptomethyl) benzoic acid and furan-2-ylmethanethiol (**5j**, **5k**). Aromatic thiols with electron-donating group exemplified by 2, 3 and 4-methoxybenzenethiol and electron-withdrawing groups were well tolerated at ortho-, meta-, para-position (**3f**, **5p** to **5s**), with the preference of electron withdrawing group for better conversions (**5n**, **5o** vs **5q**, **5s**) on the para-position of the phenyl ring. Finally, heterocyclic substrates reacted effectively (**5t**, **5u**). In conclusion, the formation of aerial disulfide was effective on substrates with a wide variety of structural characteristics. Importantly, the coupling reactions were readily scalable to 50 nmol, which was crucial for their application in DEL synthesis (see Supporting Information for details).

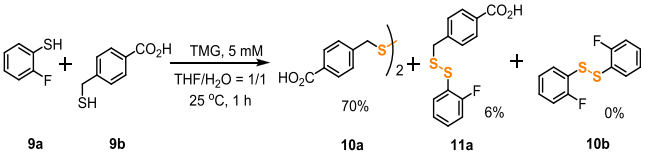
Contrary to the conventional wisdom regarding the pathway from on-DNA to off-DNA, our findings prompted us to investigate the corresponding off-DNA reaction in water under ambient conditions. Not surprisingly, the off-DNA oxidative reaction was generally challenging: The on-DNA oxidative conditions can be adapted to off-DNA with a much higher reactant concentration, but when the reactivity of a pair of thiol substrates was differentiated, symmetric disulfide products were frequently formed sequentially (Scheme 4), whereas a mixture of three products could be formed when the substrates' reactivity were comparable. Consequently, the

Scheme 3. Substrate Scope of off-DNA Thiols



[a]**1f** (5.0 nmol in H_2O, final concentration was 0.06 mM, 5.0 μL), **4** (100.0 mM in THF, final concentration was 22.22 mM, 20.0 μL), TMG (4.0 mM in THF, final concentration was 1.11 mM, 25.0 μL), V_{H2O} = 40 μL, THF/H_2O = 1/1, V_{total} = 90 μL, 25 °C, 1 h. [b]Base is TEA. [c]Conversions based on **1f**. [d]Conversions were determined by LC−MS.

Scheme 4. Off-DNA for Oxidative Disulfide Reaction



oxidative reaction yields a complex mixture when the equivalent molar ratio of thiols is utilized (see Supporting Information for details). This effort revealed that the inherently difficult chemical transformation could be easily circumvented through DNA encoded chemistry, if the chemical selectivity can be addressed under highly diluted conditions and with large excess of reagent to avoid the problems of complex side products and associated purification hurdles. This work could serve as a steppingstone to explore the chemical space which are otherwise difficult to reach, rooted on known knowledge or experience through on-DNA chemistry. Additionally, it inspired us on how we should make decision on the selection of on-DNA reaction conditions to work on initially and thus develop a workable protocol for DEL synthesis. Indeed, we often start surveys on what is known for the off-DNA reactions and decided upon our finding in the literature. This study showcases that this approach may have to evolve to accommodate new workable on-DNA chemical transformation efficiently and tackle uncharted chemical space for drug discovery.

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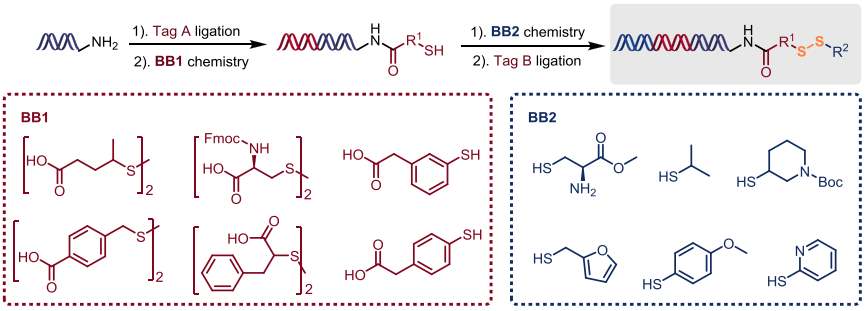
Scheme 5. A Prototype DEL Synthesis



To demonstrate the synthetic utility of this method, a prototype DEL was built under the optimized conditions (Scheme 5). This 2-Cycle, 36-member library design used 4 on-DNA symmetric disulfides and 2 free thiol substrates as Cycle-1 and 6 free thiol substrates as Cycle-2 reagents, respectively. The ligation and acylation of the bifunctional Cycle 1 reagents resulted in the formation of the desired thiols. The disulfide intermediates can be converted into free thiols easily by treatment with DTT (see Supporting Information for details). The pooled products were then exposed to the standard thiol aerial oxidization reaction conditions to generate the desired disulfide as Cycle 2 products. In subsequent assays, the N-(2-((2-fluorophenyl) disulfaneyl) ethyl)acetamide on-DNA conjugate was submitted to quantitative polymerase chain reaction analyses to determine the extent of DNA degradation during the processes. To our delight, the results indicated that the reaction conditions did not cause any evident DNA damage.

In addition, a capped substrate with no expected reactivity was subjected to the typical thiol aerial oxidization conditions as a control experiment, and the results demonstrated that the DNA oligo conjugate remained intact throughout the reaction process, thereby further verifying the DNA oligos' integrity (see Supporting Information for details).

In conclusion, we have developed a robust approach for the on-DNA oxidative disulfide reaction that features mild reaction conditions, short reaction times, and high conversions while tolerating a wide diversity of functional groups. This strategy was effective for the synthesis of new on-DNA disulfides, unveiling the uncharted chemical space for drug discovery with DELT. Also, this efficient on-DNA synthesis prompted us to design an eco-friendly off-DNA symmetrical disulfide synthesis. Future research on the substrate scope and pertinent green chemistry is considered.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03160.

Experimental details, procedures and spectral data for all new compounds are provided (PDF).

■ AUTHOR INFORMATION

Corresponding Author

Yun Jin Hu − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China;* orcid.org/0000-0001-6943-2459; Email: yunjin.hu@pharmaron.com

Authors

Lijun Xue − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Hangke Ma − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Weijie Liu − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Nicolas Duchemin − *Pharmaron UK, Ltd., Hoddesdon EN11 9FH, U.K.*

Fanming Zeng − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Jie Zhang − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Huanqing Zhang − *Pharmaron (Ningbo) Technology Development Co., Ltd., Ningbo 315336, China*

Kexin Yang − *Pharmaron Beijing Co., Ltd., Beijing 100176, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c03160

Author Contributions

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

Notes

The authors declare no competing financial interest.

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