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PII: S0040-4039(17)30146-6
DOI: http://dx.doi.org/10.1016/j.tetlet.2017.01.101
Reference: TETL 48604

To appear in: *Tetrahedron Letters*

Received Date: 10 December 2016
Revised Date: 24 January 2017
Accepted Date: 30 January 2017

Please cite this article as: Brooks, J.L., Olsen, P., Chen, L., Rodriguez, A.A., UnyLinker dimer impurity characterization and process improvement, *Tetrahedron Letters* (2017), doi: http://dx.doi.org/10.1016/j.tetlet.2017.01.101

Graphical Abstract

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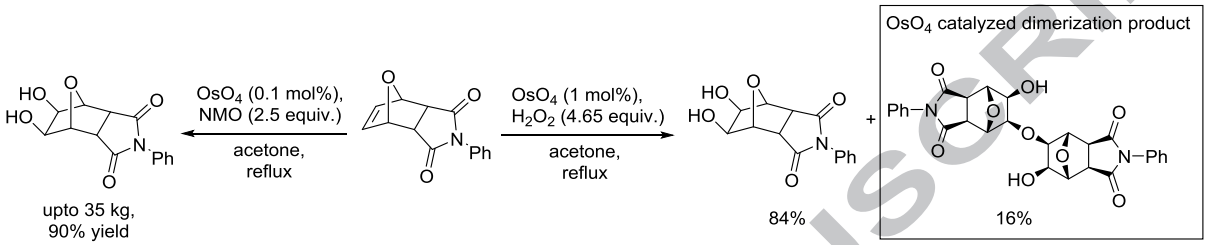

UnyLinker dimer impurity characterization and process improvement

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Highlights:

- Isolation and characterization of UnyLinker dimer
- The dimer was discovered as an impurity in oligonucleotide synthesis
- Description mechanistic proposal for osmium catalyzed intermolecular dimerization
- Process improvement that completely prevents dimer formation
- New procedure scaled to 35 kg

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Oligonucleotide
Dihydroxylation
UnyLinker
Dimerization

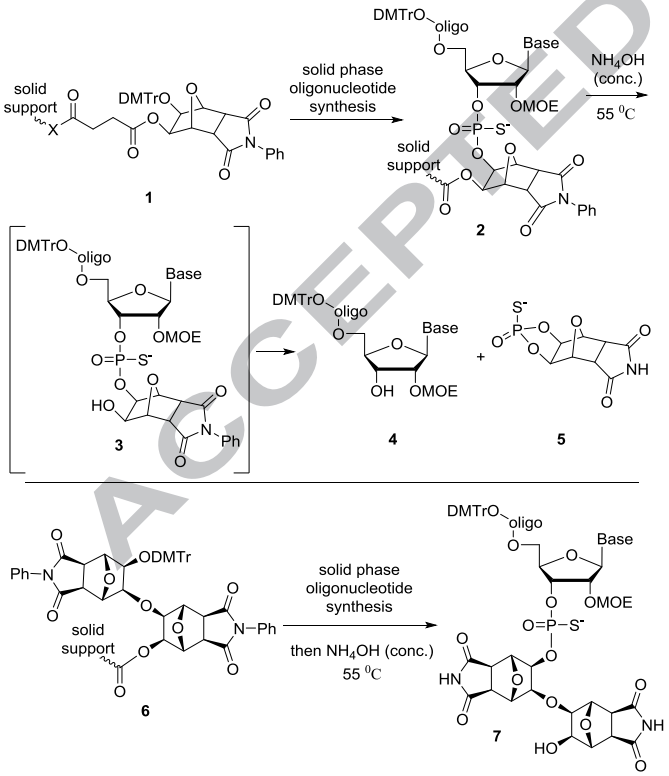
ABSTRACT

Herein, we describe the isolation and characterization of a UnyLinker dimer which, if left uncontrolled, can become incorporated in oligonucleotide products. The dimer is formed as a result of an unusual intermolecular osmium-catalyzed etherification. We demonstrate that by simply replacing H_2O_2 as the co-oxidant with NMO, none of the UnyLinker dimer impurity is formed.

Synthetic oligonucleotides are a novel class of drug[1] with the potential to treat a wide range of illnesses including cancer, cardiovascular and metabolic conditions, neurological disorders, and ophthalmic diseases. Oligonucleotides designed to interact with a variety of molecular targets including coding and non-coding RNA and proteins are being investigated with 140 clinical trials currently listed on ClinicalTrials.gov. Five oligonucleotide drugs have reached the market.

As part of an ongoing investigation into oligonucleotide impurities generated during the manufacturing process [2] we report here the isolation, characterization, and elimination of a previously unknown impurity of the universal linker molecule (UnyLinker®) that is commonly used in solid phase oligonucleotide synthesis. The UnyLinker impurity leads to multiple oligonucleotide impurities that are difficult to remove. Therefore, its elimination results in higher purity oligonucleotide products.

Our oligonucleotide manufacturing process is performed using an optimized version of the phosphoramidite approach.[2] We utilize a solid-support with UnyLinker **1** attached to the polymer-matrix (Scheme 1).[3] The use of UnyLinker eliminates the need to load the solid-support with the first nucleotide, thereby enabling the synthesis of any oligonucleotide with a single solid-support, regardless of the identity of its 3′-nucleotide. Upon completion of synthesis, solid-support-bound oligonucleotide **2** is subjected to ammonolysis to cleave UnyLinker from the resin, forming intermediate **3**. The newly unmasked alcohol attacks the 3′-thiophosphate to release oligonucleotide **4** and UnyLinker cyclic thiophosphate **5**. Byproducts from synthesis, including hydrolyzed Unylinker fragments, are removed during the subsequent chromatographic purification step.



Scheme 1: Solid phase oligonucleotide synthesis with UnyLinker and UnyLinker dimer.

During a recent analysis of an oligonucleotide, new impurities were observed corresponding to mass increases of 458, 476, and 494 Daltons and totaling 0.64% of the product. The masses were consistent with an oligonucleotide attached at the 3′-end to a dimeric form of the UnyLinker, **7** (Figure 1). Unlike UnyLinker, the dimer likely fails to cleave from the oligonucleotide because the attacking hydroxyl group is blocked as the ether linkage (Scheme 1).

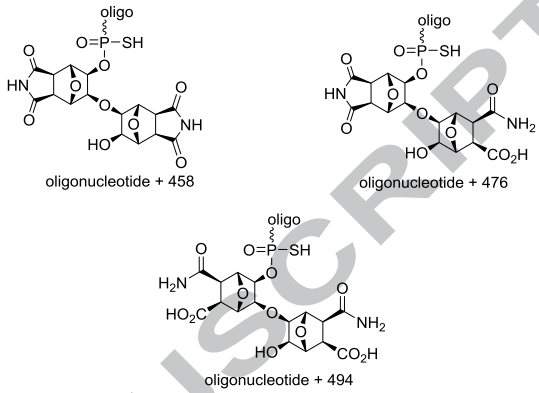
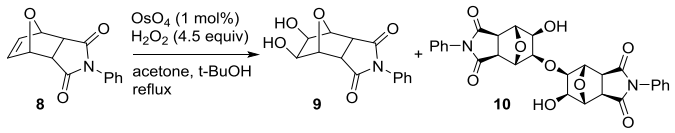


Figure 1: Proposed structures of UnyLinker dimer impurities showing relative stereochemistry.

Examination of the batch of UnyLinker used to synthesize the oligonucleotide revealed that it indeed contained a dimeric species. It was determined that the dimer formed during the osmium tetroxide dihydroxylation of olefin **8**, resulting in desired diol **9** and the dimeric ether **10** in 84:16 ratio and 94% yield (Scheme 2).[4] The dimer **10** was isolated from a crude reaction mixture, fully characterized by NMR,[5] and the structure confirmed by X-ray crystal analysis of the 4-nitro-benzyl ester analog (Figure 2).



Scheme 2: Synthesis of diol **9** and dimer **10**

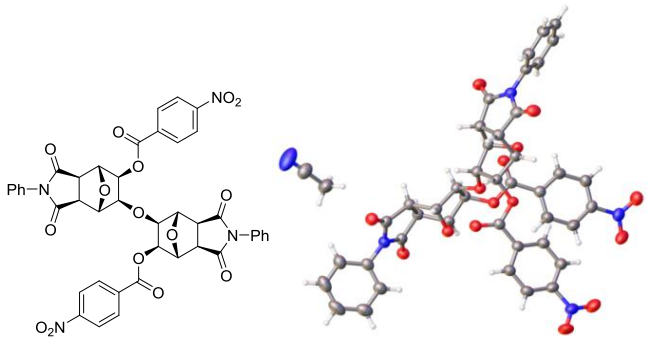
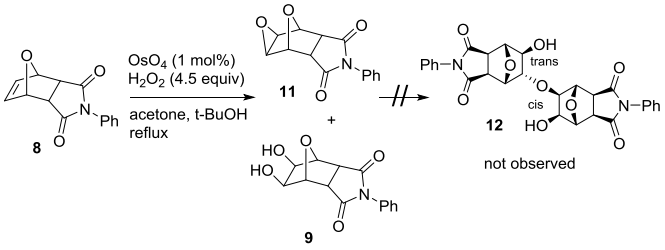


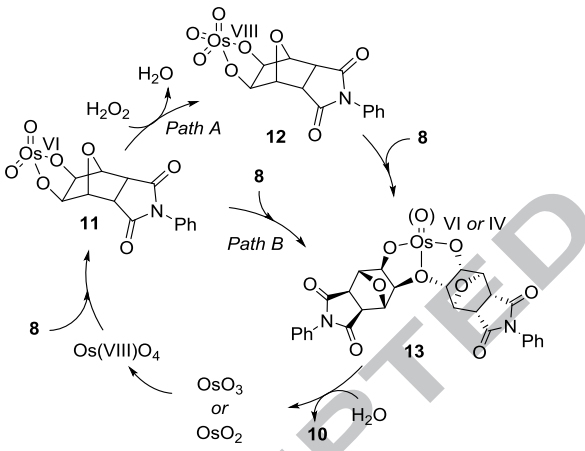
Figure 2: X-ray crystal structure of nitrobenzyl adduct

The mechanistic origin of this unusual dimer requires additional explanation. One possibility is that olefin **8** undergoes epoxidation to **11** and subsequent ring opening by attack of diol **9** (Scheme 3). This was ruled out however, as neither epoxide **11** or cis-trans product **12**, i.e. the product of attack of **9** on **11**, were observed. Furthermore, when epoxide **11** was prepared and subjected to reaction conditions (from Scheme 2) with diol **9**, no dimer was formed.



Scheme 3: Proposed epoxide opening resulting in dimer formation

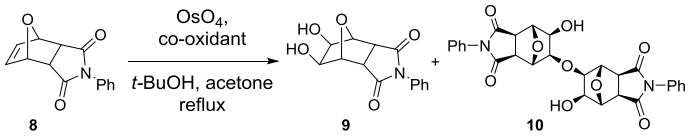
A second possibility is that **10** results from intermolecular osmium-catalyzed dimerization. While several examples of osmium-catalyzed intramolecular etherifications are known,[6, 7] to the best of our knowledge, the intermolecular variant has not been reported. In particular, we propose that osmate ester **11** (Scheme 4) can either be hydrolyzed to form the diol **9,** or oxidized to the Os(VIII) intermediate **12** by H_2O_2 (*Path A*). A second equivalent of olefin **8** can add to the osmate ester **12** resulting in **13**, which is hydrolyzed to dimer **10** and OsO_3. A second possibility (*Path B*) is that Os(VI) intermediate **11** reacts directly with olefin **8** resulting in the Os(IV) oxidation state of **13**,[8] which is subsequently hydrolyzed and reoxidized to give dimer **10** and OsO_2.



Scheme 4: Catalytic mechanism for formation of dimer **10**

To address the problem of dimer formation and to improve the dihydroxylation, a brief screening of reaction conditions was conducted (Table 1). As noted, the use of H_2O_2 as a co-oxidant led to dimer formation (entry 1). Similar results were obtained with *t*BuO_2H (entry 2), albeit with diminished yield. By switching the co-oxidant to 4-methylmorpholine *N*-oxide (NMO, entry 3), no dimer product was observed, and the reaction rate was increased (from 8 to 4h reaction time, entry 3)[4]. We were able to reduce the amount of co-oxidant to 2.5 equivalents and reduce the catalyst loading to 0.1 mol% without affecting the conversion or reaction time (entries 4 and 5). However, when the catalyst loading was reduced to 0.01 mol%, the reaction was too slow to be practicable (24h, entry 6). Our best reaction conditions[9] were repeated at a 35 kg scale, and resulted in 90% isolated yield of diol **9** without formation of dimer **10**.

Table 1: Improved synthesis of UnyLinker diol **9**[a]



Entry	OsO_4 (mol%)	Reaction time (h)	Co-oxidant	Product Ratio (**9**:**10**)	% yield
1	1	8	H_2O_2	84:16	94
2	1	8	*t*BuO_2H	88:12	45
3	1	4	NMO	100:0	90
4[b]	1	4	NMO	100:0	90
5[b]	**0.1**	**4**	**NMO**	**100:0**	**90**
6[b]	0.01	24	NMO	100:0	89
7[b,c]	0.1	4	NMO	100:0	90[d]

[a.] Reaction conditions: 10.0 g (41.5 mmol) olefin suspended in 100 mL acetone, and co-oxidant added (4.65 equiv) followed by OsO_4 as a 20 mM solution in *t*BuOH. The solution was heated to reflux, behind a blast shield, until all of the starting material was consumed (TLC), typically 8h. [b.] 2.5 equiv. NMO added. [c.] The reaction was preformed starting from 35 kg olefin. [d.] Isolated yield.

In conclusion, we have identified and characterized a new oligonucleotide impurity resulting from a Unylinker dimer. The dimer likely resulted from an unusual intermolecular osmium-catalyzed etherification during the dihydroxylation step of Unylinker synthesis and was formed as a result of an osmate ester intermediate reacting with a second equivalent of olefin. We demonstrated that by changing the co-oxidant used during the dihydroxylation from H_2O_2 to NMO, we can prevent formation of the dimer while reducing catalyst loading and increasing yield. This new method is currently used to produce dimer-free UnyLinker at large scale.

Acknowledgments

We sincerely thank Herbert Brown Pharmaceutical Research & Laboratories (India), Daniel Capaldi and Andy McPherson (Ionis) for helpful discussions, and Arnold Rheingold (UCSD) for X-ray crystallography services.

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[4] For representative HPLC trace see supporting information.

[5] **^{1}H NMR** (300 MHz) δ 7.55-7.38 (m, 6H), 7.20 (d, *J* = 6.0 Hz, 4H), 4.92 (broad s, 2H), 4.78 (s, 2H), 4.40 (s, 2H), 4.07 (d, *J* = 7.0 Hz, 2H), 3.94 (d, *J* = 7.0 Hz, 2H), 3.15 (q, *J* = 74.5 Hz, 4H). **^{13}C NMR** (75 MHz) δ 176.0, 132.2, 128.9, 126.7, 84.8, 82.0, 80.6, 73.0, 45.8.

[6] For review see: a) Pilgram, B. S.; Donohoe, T. J. *J. Org. Chem.* **2013**, *78*, 2149. b) Piccialli, V. *Synthesis* **2007**, *17*, 2585

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[9] The alkene **6** (10.00 g, 41.45 mmol) was suspended in 85 mL of acetone, and a solution of 50% aqueous NMO (2.5 equiv., 21.49 mL, 103.6 mmol) was added resulting in a biphasic slurry of alkene. A solution of OsO_4 in *t*BuOH (0.02 M, 2.07 mL, 0.001 equiv.) was added and the reaction mixture brought to reflux. As the solution begins to reflux the layers coalesce and the alkene fully dissolves (~5 min.); the diol then precipitates from solution as it is formed. Reflux is continued until the starting material is consumed at which point the solution is cooled to room temperature, quenched with saturated aqueous sodium thiosulfate (50 mL), and the diol collected by filtration. The solid was washed with H_2O (50 mL) and acetone (100 mL), collected, and dried under vacuum to afford diol **9** (10.22 g, 90% yield).
^{1}H NMR (300 MHz) δ 7.55-7.38 (m, 3H), 7.25-7.18 (m, 2H), 5.03 (s, 2H), 4.41 (s, 2H), 3.97 (s, 2H), 3.17 (s, 2H). **^{13}C NMR** (75 MHz) δ 176.2, 132.2, 128.9, 128.4, 126.8, 84.1, 71.8, 45.5.