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Intrastrand locks increase duplex stability and base pairing selectivity†

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Oligodeoxynucleotide probes with disulfide locks between neighboring nucleobases show increases in melting point for duplexes with RNA target strands of up to 7.6 °C. The weakly pairing TT dimers are replaced with locked 2′-deoxy-5-(thioalkynyl)uridine residues *via* automated synthesis.

The affinity of oligonucleotides for target strands is weaker than the number of base pairs may suggest, due to a large entropy–enthalpy compensation during duplex formation.[1] Hydrogen bonding, stacking, and other energetically favorable interactions are compensated by a loss in translation, conformational, and vibrational degrees of freedom, so that the bulk of the binding enthalpy is not translated into a gain in free energy. The large number of conformers accessible to oligonucleotides also reduces sequence selectivity. Non-Watson–Crick pairing is compatible with the backbone of DNA, making duplex formation less predictable than desirable for hybridization probes binding a single target in a genomic context. The likelihood of mismatch formation is greatest at the termini, where fraying and wobbling is common.[2] Restricting the conformational flexibility of oligonucleotide probes to the conformation found in the desired duplex should increase target affinity and selectivity.

The concept of rigidifying oligonucleotides to gain target affinity has been demonstrated impressively for nucleosides with covalent links that lock a desired conformation of the ribose (Fig. 1a). Bicyclo-[3] or tricyclo-DNA,[4] and locked nucleic acids (LNA)[5] are among the most successful classes of modified oligonucleotides in biology. Cross links between strands (Fig. 1c) are detrimental for cells, but synthetic constructs, such as methylene bridged base pairs,[6] metal–salen complexes,[7] bis-maleimides,[8] or triazolides,[9] produce valuable bioorganic compounds. For triplexes, interstrand cross links and links in circular DNA can increase stability and sequence selectivity.[10]

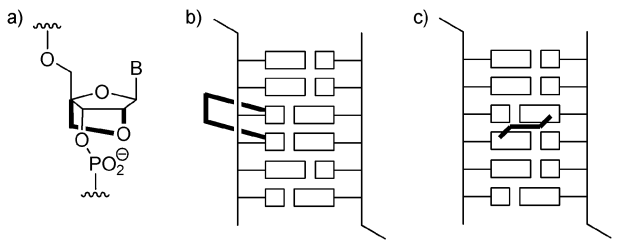

Fig. 1 Locks for nucleic acids (bold lines). (a) Locked nucleoside (LNA), (b) intrastrand-lock, and (c) interstrand cross link.

A third possible approach involves intrastrand locks between nucleotides of a hybridization probe (Fig. 1b). Experimental realizations are rare.[11] We were interested in the effect of intrastrand locks on hybridization probes. We chose disulfide links between neighboring nucleotides, assuming that they would form spontaneously in the presence of air.[12–14] We replaced TT dinucleotides as the most weakly pairing dinucleotide, as a step towards isostable duplexes for high fidelity detection in massively parallel fashion.[15,16] High fidelity is very important for probes binding short RNA species, such as microRNAs,[17] that are difficult to amplify by PCR.[18]

The intrastrand locks were introduced at the 5-position of 2′-deoxyuridine residues *via* butynyl and hexynyl linkers (Scheme 1). Other oligodeoxynucleotides with 2′-deoxyuridines as the site of disulfide cross linking are known.[10,19] Alkynyl substituents at the 5-position point into the major groove of duplexes,[20–23] where they provide additional stacking interactions.[24]

Phosphoramidites **1** and **2** were prepared following a route for related compounds.[10a] The route of Benner and coworkers[25] gave lower yields. Silyl-protected 5-iodo-2′-deoxyuridine (**3**) was cross-coupled to the alkynyl alcohol, and the resulting deoxyuridines (**4** or **5**) were mesylated and reacted with thiobenzoate to give protected thioalkynyldeoxyuridines **6** and **7**. Desilylation with the known mixture of HF·Py and TBAF[10a] gave **8**/**9**, and dimethoxytritylation gave **10**/**11**. Phosphitylation produced phosphoramidites **1** and **2** in 30% and 44% overall yield, respectively. Oligodeoxynucleotides were assembled *via* automated solid-phase synthesis. Pre-treatment with NEt_3 was followed by full deprotection and cleavage from the support with NH_3, $MeNH_2$, and DTT.[10a,26] Intrastrand locks formed upon diluting with buffer (pH 5) and exposure to air. Remaining cyanoethyl adducts were removed by HPLC, giving **12–22** in up to 22% yield. Dithiol and disulfide differ in mass by just 2 Da, so MALDI spectra were also measured in reflectron mode with internal standard to confirm structures (Fig. S22–S43, ESI†).

Self-complementary control duplex $(5'\text{-TTGCGCAA-}3')_2$ $(\mathbf{23})_2$ and locked duplexes $(\mathbf{12})_2$–$(\mathbf{14})_2$ were subjected to UV-melting. The latter showed an increase in melting point

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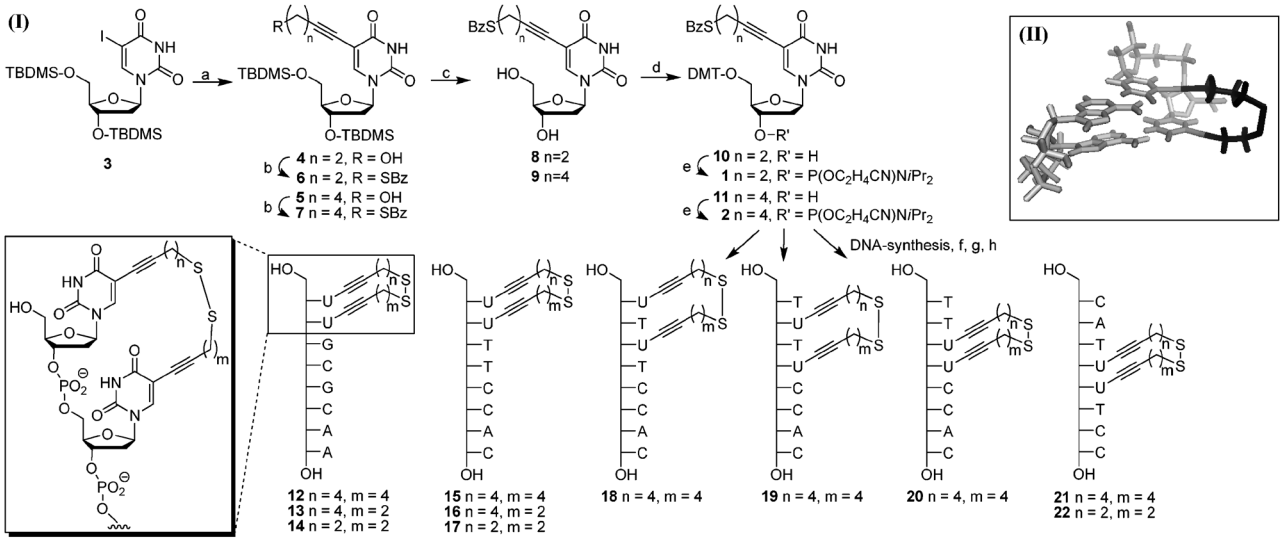

[a] Conditions: (a) 3-Butyn-1-ol or 5-hexyn-1-ol, CuI, NEt_3, $Pd(PPh_3)_4$, DMF, 53-72%; (b) 1. NEt_3, CH_3SO_2-Cl, DMF, -55 °C; 2. PhCOSH, RT, 85-90%; (c) HF · Py, TBAF, pyridine, 78-82%; (d) DMT-Cl, pyridine, 86-88%; (e) $NC(CH_2)_2O$-P(N*i*Pr$_2$)Cl, DIEA, CH_3CN, 94-99%;(f) NEt_3, DMF; (g) $NH_{3(aq)}$, $MeNH_2$, DTT; (h) air (O_2), phosphate buffer, pH 5.

Scheme 1 (I) Synthesis of intrastrand-locked oligodeoxynucleotides **12–21**.[a] (II) Structure of intrastrand-locked d($U^{S4}U^{S2}$):*r*(AA) dimer duplex, as generated by molecular modeling for A-form geometry using Macromodel.

of 8.6–13.7 °C (4.3–6.9 °C per lock, Table 1). The short-hand for a residue with an ethylene unit in the side chain is U^{S2}, and for a residue with a butylene chain it is U^{S4}. The mixed construct with a lock containing one U^{S2} and one U^{S4} gave the largest ΔT_m. Next, we studied non-self complementary sequence 5′-TTTTCCAC-3′ (**24**) with DNA and RNA targets. A total of six locks was introduced at different positions (**15–20**, Scheme 1). The highest UV-melting point was found for RNA as a target, with +7.6 °C for the U^{S2}/U^{S4} lock at the terminus of probe (**16**:**25**).

Much smaller increases in T_m were found for locks bridging non-neighboring residues (**18**:**25** and **19**:**25**), and a long lock in the middle of the duplex (**20**:**25**). When DNA was the target (**26**), slightly lower melting point increases were found, and the shortest of the links gave the strongest effect (+7.0 °C, **17**:**26**). Locks clamping a base triplet gave melting point depressions of 2.2–2.4 °C (Table 1).

A third sequence motif containing the locks in the middle of the strand was based on 5′-CCTTTTAC-3′ (**27**). Melting points of **21** and **22** with both RNA and DNA target strands showed that the short lock with two ethylene units flanking the disulfide is not well tolerated in the interior of the helix. Locks with butylene chains gave melting point increases, though, with the higher ΔT_m for the duplex with the RNA target (+4.2 °C; **21**:**28**).

Next, we studied base pairing selectivity by measuring UV-melting points of duplexes with mismatched bases opposite locks (Fig. S44, ESI†, Table 2). In every single case, the locked strands showed greater selectivity than their unmodified counterparts. For locked strands **21** and **22**, melting points with such targets were even <10 °C. The effect is pronounced at the terminus, where the control duplex (**24**:**30**) gives barely any melting point depression for a T:U mismatch (−1.7 °C). Increases in ΔT_m over that of the control were also found for the penultimate position and the third position from the terminus. When DNA is the target, mismatch discrimination is greater to begin with, but increases further in the presence of the lock. For the butylene/butylene lock, a ΔT_m of almost 10 °C for a T:T mismatch at the very terminus was found, a value exceeding that induced by the best commercial fidelity-enhancing cap.[27]

Table 1 UV melting points with perfect match target strands

Target strand	Probe strand	T_m[a]/°C	ΔT_m[b]/°C
	23[c]	41.9 ± 0.6	—
	12[c]	53.0 ± 0.5	+11.1/5.6[d]
	13[c]	55.6 ± 0.9	+13.7/6.9[d]
	14[c]	50.5 ± 0.9	+8.6/4.3[d]
r(GUGGAAAA) (**25**)			
	24	30.3 ± 0.5	—
	15	36.7 ± 0.5	+6.4
	16	37.9 ± 0.6	+7.6
	17	37.7 ± 0.6	+7.4
	18	31.9 ± 0.6	+1.6
	19	33.5 ± 0.5	+3.2
	20	31.8 ± 0.5	+1.5
GTGGAAAA (**26**)			
	24	24.9 ± 0.4	—
	15	31.3 ± 0.8	+6.4
	16	30.4 ± 0.6	+5.5
	17	31.9 ± 0.4	+7.0
	18	22.7 ± 0.9	−2.2
	19	22.5 ± 0.9	−2.4
	20	27.8 ± 0.8	+2.9
r(GUAAAAGG) (**28**)			
	27	24.8 ± 0.5	—
	21	29.0 ± 0.7	+4.2
	22	23.5 ± 1.7	−1.3
GTAAAAGG (**29**)			
	27	23.8 ± 1.0	—
	21	26.2 ± 0.5	+2.4
	22	19.0 ± 0.6	−4.8

[a] Average of 4 curves ± SD 1.5 μM strand concentration and 1 M NaCl, 10 mM phosphate buffer, pH 7.0. [b] ΔT_m to unmodified control duplex. [c] Self-complementary sequence; no separate target strand. [d] ΔT_m per lock.

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Table 2 UV melting points of duplexes containing U:U or U:T mismatches. Mismatched bases are given in boldface

Target strand	Probe strand	T_m[a]/°C	ΔT_m[b]/°C
r(GUGGAAA**U**) (**30**)			
	24	28.6 ± 0.6	−1.7
	15	30.3 ± 1.0	−6.4
	16	31.9 ± 0.5	−6.0
	17	32.9 ± 0.7	−4.8
	18	28.6 ± 0.7	−3.3
r(GUGGAA**U**A) (**31**)			
	24	27.0 ± 0.3	−3.3
	19	27.9 ± 0.3	−5.6
r(GUGGA**U**AA) (**32**)			
	24	20.7 ± 0.4	−9.6
	20	21.4 ± 0.8	−10.4
GTGGAAA**T** (**33**)			
	24	21.5 ± 0.8	−3.4
	15	21.7 ± 1.4	−9.6
	16	22.9 ± 1.0	−7.2
	17	22.8 ± 1.8	−9.2
	18	19.1 ± 0.8	−3.6
GUGGAA**T**A (**34**)			
	24	18.0 ± 1.0	−6.9
	19	14.4 ± 0.4	−8.1
GUGGA**T**AA (**35**)			
	24	11.2 ± 1.3	−13.7
	20	11.1 ± 1.9	−16.7

[a] Average of 4 curves ± SD 1.5 μM strand concentration and 1 M NaCl, 10 mM phosphate buffer, pH 7.0. [b] Melting point difference to perfect match control duplex (compare Table 1).

Molecular modeling suggests that the ethylene/butylene combination can form an unstrained link (Scheme 1–II), and so do CD spectra of duplexes of **16** with **25** and **26** (ESI†, Fig. S45–S46). Thermodynamic data show that the stabilizing effect is indeed of entropic origin (Table S1, ESI†). Covalently locked neighboring nucleotides are most stabilizing at the very terminus, where pairing is weak and wobbling is pronounced. This is also where the fidelity-enhancing effect is most desirable, to overcome the poor discriminating ability of unmodified probes.

Disulfides have several advantages over other locking chemistries, such as reductive amination and amide formation that were also tested in the early phase of our study. Firstly, there is no need for additional synthetic steps. The lock forms spontaneously after automated DNA synthesis and deprotection. Secondly, there is excellent orthogonality to other functional groups, and no side reactions must be feared, other than alkylation and intermolecular locking, which appears to compete poorly with intramolecular locking under our conditions. Thiols also have minimal steric demand, making it easy to close very large rings, and disulfide formation has a large free energy driving force, avoiding incomplete reactions. Still, disulfide locking can be reversed through reduction. Exploratory melting curves with duplex **16**:**25** show a T_m drop of 5.3 °C when DTT is added. The T_m of the duplex of **18**, with its poor three nucleotide lock, and **25** increased by 1.5 °C upon addition of DTT.

In conclusion, we have demonstrated that intrastrand locks of proper length elevate the melting point of duplexes with DNA and RNA target strands. The locks can be introduced through DNA synthesis with phosphoramidite building blocks and a two-step deprotection. Intrastrand-locked nucleic acids may find use in biomedicine where high fidelity hybridization is required. We are currently studying other nucleobases and nucleotide distances.

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Notes and references

1 (*a*) G. Vesnaver and K. J. Breslauer, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 3569–3573; (*b*) M. S. Searle and D. H. Williams, *Nucleic Acids Res.*, 1993, **21**, 2051–2056.
2 (*a*) D. Andreatta, S. Sen, J. L. Pérez Lustres, S. A. Kovalenko, N. P. Ernsting, C. J. Murphy, R. S. Coleman and M. A. Berg, *J. Am. Chem. Soc.*, 2006, **128**, 6885–6892; (*b*) K.-W. Tsai, W.-C. Chan, C.-N. Hsu and W.-C. Lin, *BMC Mol. Biol.*, 2010, **11**, 34–42.
3 (*a*) M. Tarkoy, M. Bolli, B. Schweizer and C. J. Leumann, *Helv. Chim. Acta*, 1993, **76**, 481–510; (*b*) A. Stauffinger and C. J. Leumann, *Eur. J. Org. Chem.*, 2009, 1153–1162.
4 (*a*) R. Steffens and C. J. Leumann, *J. Am. Chem. Soc.*, 1997, **119**, 11548–11549; (*b*) D. Ittig, A. B. Gerber and C. J. Leumann, *Nucleic Acids Res.*, 2011, **39**, 373–380.
5 (*a*) S. K. Singh, P. Nielsen, A. A. Koshkin and J. Wengel, *Chem. Commun.*, 1998, 455–456; (*b*) A. A. Koshkin, P. Nielsen, M. Meldgaard, V. K. Rajwanshi, S. K. Singh and J. Wengel, *J. Am. Chem. Soc.*, 1998, **120**, 13252–13253.
6 X. Qiao and Y. Kishi, *Angew. Chem., Int. Ed.*, 1999, **38**, 928–931.
7 K. V. Gothelf, A. Thomsen, M. Nielsen, E. Cló and R. S. Brown, *J. Am. Chem. Soc.*, 2004, **126**, 1044–1046.
8 R. Tona and R. Häner, *Mol. BioSyst.*, 2005, **1**, 93–98.
9 A. H. El-Sagheer, R. Kumar, S. Findlow, J. M. Werner, A. N. Lane and T. Brown, *ChemBioChem*, 2008, **9**, 50–52.
10 (*a*) N. C. Chaudhuri and E. T. Kool, *J. Am. Chem. Soc.*, 1995, **117**, 10434–10442; (*b*) E. T. Kool, *Chem. Rev.*, 1997, **97**, 1473–1487.
11 (*a*) A. Kumar, *E-J. Chem.*, 2011, **8**, 507–512; (*b*) Y. Liu, R. Wang, L. Ding, R. Sha, P. S. Lukeman, J. W. Canary and N. C. Seeman, *ChemBioChem*, 2008, **9**, 1641–1648.
12 A. E. Ferentz and G. L. Verdine, *J. Am. Chem. Soc.*, 1991, **113**, 4000–4002.
13 J. Milton, B. A. Connolly, T. T. Nikiforov and R. Cosstick, *J. Chem. Soc., Chem. Commun.*, 1993, 779–780.
14 (*a*) G. D. Glick, *J. Org. Chem.*, 1991, **56**, 6746–6747; (*b*) G. D. Glick, *Biopolymers*, 1998, **48**, 83–96.
15 C. Ahlborn, K. Siegmund and C. Richert, *J. Am. Chem. Soc.*, 2007, **129**, 15218–15232.
16 K. Siegmund, C. Ahlborn and C. Richert, *Nucleosides, Nucleotides Nucleic Acids*, 2008, **27**, 376–388.
17 D. Baek, J. Villén, C. Shin, F. D. Camargo, S. P. Gygi and D. P. Bartel, *Nature*, 2008, **455**, 64–71.
18 A. W. Wark, H. J. Lee and R. M. Corn, *Angew. Chem., Int. Ed.*, 2008, **47**, 644–652.
19 (*a*) J. T. Goodwin and G. D. Glick, *Tetrahedron Lett.*, 1993, **34**, 5549–5552; (*b*) S. E. Osborne, R. J. Cain and G. D. Glick, *J. Am. Chem. Soc.*, 1997, **119**, 1171–1182.
20 T. Kottysch, C. Ahlborn, F. Brotzel and C. Richert, *Chem.–Eur. J.*, 2004, **10**, 4017–4028.
21 T. J. Bandy, A. Brewer, J. R. Burns, G. Marth, T. Nguyen and E. Stulz, *Chem. Soc. Rev.*, 2011, **40**, 138–148.
22 M. Ahmadian, P. Zhang and D. E. Bergstorm, *Nucleic Acids Res.*, 1998, **26**, 3127–3135.
23 I. Luyten and P. Herdewijn, *Eur. J. Med. Chem.*, 1998, **33**, 515–576.
24 (*a*) T. W. Barnes III and D. H. Turner, *J. Am. Chem. Soc.*, 2001, **123**, 4107–4118; (*b*) B. M. Znosko, T. W. Barnes III, T. R. Krugh and D. H. Turner, *J. Am. Chem. Soc.*, 2003, **125**, 6090–6097.
25 (*a*) H. A. Held, A. Roychowdhury and S. A. Benner, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 391–404; (*b*) H. A. Held and S. A. Benner, *Nucleic Acids Res.*, 2002, **30**, 3857–3869.
26 F. Hofbauer and I. Frank, *Chem.–Eur. J.*, 2010, **16**, 5097–5101.
27 Z. Dogan, R. Paulini, J. A. Rojas Stütz, S. Narayanan and C. Richert, *J. Am. Chem. Soc.*, 2004, **126**, 4762–4763.

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