Modified Nucleotides

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Structure-Based Design of a Highly Constrained Nucleic Acid Analogue: Improved Duplex Stabilization by Restricting Sugar Pucker and Torsion Angle γ**

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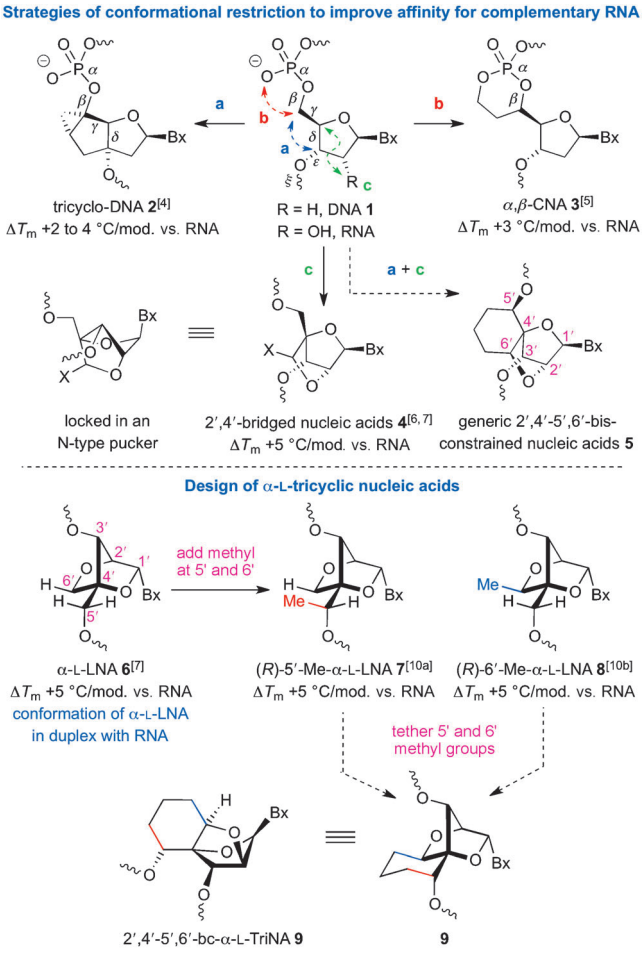
Oligonucleotide modifications, which enhance affinity for cognate RNA, have been used extensively as antisense agents[1] and for oligonucleotide-based diagnostic applications.[2] In general, modifications that improve affinity by conformational restriction of the sugar–phosphate backbone have been the most successful, because they do not interfere with the specificity of Watson–Crick base pairing and can be used across a wide range of oligonucleotide sequences with predictable results.[1,3] Over the past two decades, at least three distinct successful strategies for incorporating covalent conformational constraints into natural nucleic acids (NAs) have been described. The first strategy restricts rotation around torsion angles γ and δ in 2′-deoxynucleotide subunits (**1**), as exemplified by the tricyclo-DNA (tcDNA **2**) scaffold reported by Leumann.[4] A second strategy reported by Escudier[5] improves affinity for RNA and DNA complements by restricting backbone torsion angles α and β into the canonical *ap* and *-sc* range found in A-form DNA duplexes (α,β-constrained nucleic acid or α,β-CNA **3**). The third, and perhaps the most successful strategy, involves locking the nucleoside furanose ring in an N-type (northern) sugar pucker, as exemplified by 2′,4′-bridged (locked) nucleic acids **4**.[6,7]

While each of the above approaches have been explored individually, incorporating more than one type of conformational constraint into a single modification could provide incremental increases in the binding affinity of a modified oligonucleotide that may be unattainable through a single mode of constraint. In this Communication, we report a new, highly constrained oligonucleotide modification analogous to that represented by the generic formula of **5** (Scheme 1), which improves oligonucleotide duplex thermostability by locking the furanose sugar pucker, as well as restricting rotation around torsion angle γ.



Scheme 1. Rationale for the design of α-L-tricyclic nucleic acids.

We chose α-L-*ribo*-configured locked nucleic acid (α-L-LNA) **6** as the starting point to evaluate our strategy of dual conformational restriction (Scheme 1). α-L-LNA (**6**) exhibits LNA-like high-affinity recognition of complementary nucleic acids when incorporated in appropriate oligonucleotide sequences.[7] The increased affinity of α-L-LNA-modified oligonucleotides for RNA stems from a combination of locking the sugar furanose ring in an N-type conformation along with the α-L-configuration of the nucleoside monomer.[8] Structural studies of an α-L-LNA-modified DNA/RNA

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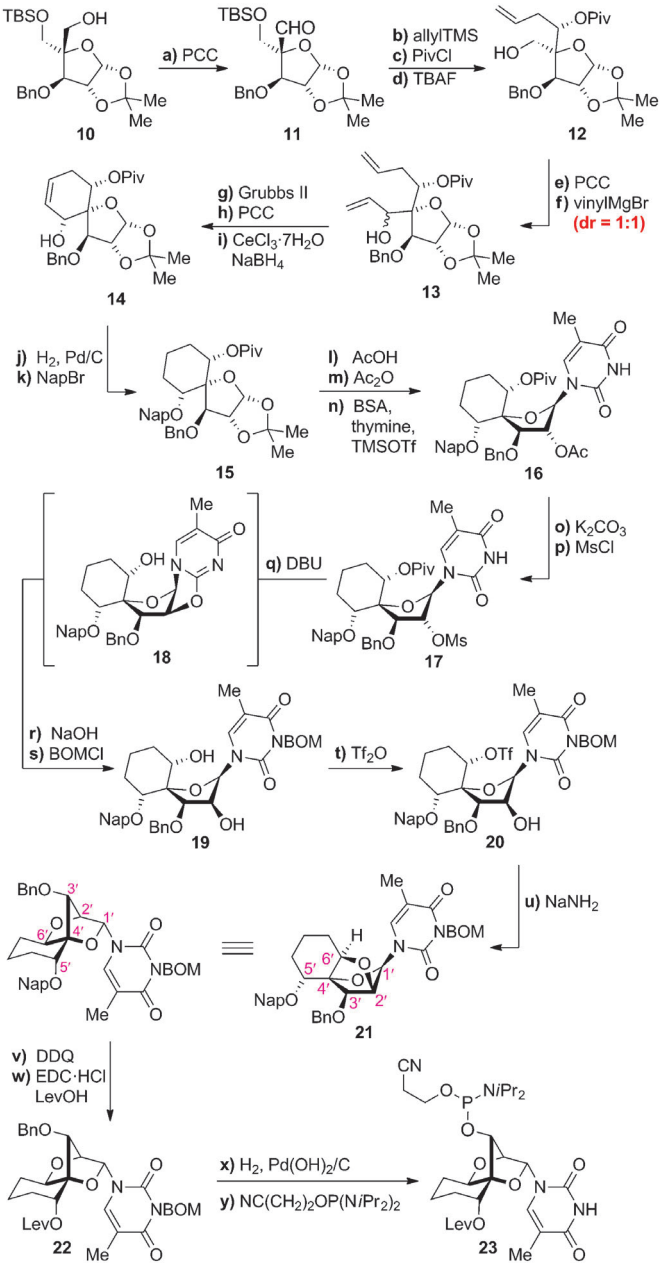
duplex showed that the 2′,4′-bridge of α-L-LNA lies inside the major groove and also mapped the orientation of torsion angle γ for the modified nucleotide within the oligonucleotide duplex.[9] Using the NMR structure of the modified duplex as a starting point, we previously introduced (*R*)-configured methyl groups at the 5′- and 6′-positions on the α-L-LNA scaffold to give analogues **7** and **8**, respectively.[10] Evaluation of oligonucleotides modified with **7** or **8** in thermal denaturation experiments revealed that both of these analogues display α-L-LNA-like affinity for RNA. Accordingly, we hypothesized that tethering these methyl groups together, forming a six-membered ring between the 5′- and 6′-positions of the α-L-LNA nucleoside monomer (that is 2′,4′-5′,6′-bis-constrained-α-L-tricyclic nucleic acid (**9**) or 2′,4′-5′,6′-bc-α-L-TriNA), would further improve affinity for RNA by restricting rotation around angle γ.

While considering various approaches to **9**, we were aware that stereocontrolled formation of the 2′,4′-anhydro bridge (that is the 1,4-dioxa[2.2.1]heptane motif) in **9** would present a major synthetic challenge, and thus planned the route accordingly. Oxidation of **10**, readily available from diacetone-D-glucose,[11] afforded aldehyde **11**, which was subjected to a Sakurai allylation reaction[12] to give **12** after pivaloylation and removal of the TBS protecting group (Scheme 2). Oxidation of the α-configured hydroxymethyl group to the corresponding aldehyde and subsequent treatment with vinylmagnesium bromide afforded a 1:1 mixture of allylic alcohols **13**, which were converted into the spirocyclic cyclohexene using a ring-closing metathesis reaction in the presence of Grubbs' second-generation catalyst and in excellent overall yield. An oxidation–reduction sequence afforded enantiopure spirocycle **14**, which was subjected to catalytic hydrogenation and protected as the 2-naphthylmethyl (Nap) ether to give **15**. A three-step sequence culminating with a Vorbrüggen glycosylation afforded orthogonally *O*-protected spirocyclic thymidyl nucleoside **16** in 80% overall yield.[13] Chemoselective ester hydrolysis, followed by mesylation of the 2′-OH group gave mesylate **17**.

In the original synthetic proposal for **9**, we envisaged that exposing the 2′,6′-dimesylate analogue of **17** to hydroxide would facilitate formation of the 2′,4′-anhydro bridge in a single synthetic step, however, formation of the 2,6′-anhydronucleoside prevailed instead. These initial attempts at constructing the 2′,4′-anhydro bridge of **9** indicated that it was essential to protect the free nitrogen atom of **17** prior to cycloetherification. Accordingly, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU)-induced formation of the 2,2′-



Scheme 2. Synthesis of thymidyl spironucleoside **23**.[14] Reagents and conditions: a) pyridinium chlorochromate (PCC), NaOAc, 4 Å molecular sieves (MS), CH_2Cl_2, 0°C to RT, 5 h; b) $BF_3{\cdot}OEt_2$, allyltrimethylsilane, CH_2Cl_2, −78°C, 3 h, 78% over two steps; c) pivaloyl chloride (PivCl), 4-dimethylaminopyridine (DMAP), pyridine, 100°C, 24 h, 92%; d) tetra-*n*-butylammonium fluoride (TBAF), THF, RT, 3 h, 88%; e) PCC, NaOAc, 4 Å MS, CH_2Cl_2, 0°C to RT, 3 h, 81%; f) vinylmagnesium bromide, Et_2O, 0°C, 30 min., 95% (dr = 1:1); g) Grubbs II (1 mol%), CH_2Cl_2, 40°C, 1 h, 93%; h) PCC, NaOAc, 4 Å MS, CH_2Cl_2, 0°C to RT, 4 h; i) $CeCl_3{\cdot}7H_2O$, $NaBH_4$, MeOH, 0°C, 20 min., 85% over two steps; j) H_2, 10% Pd/C, THF, RT, 4 h, 96%; k) NapBr, NaH, tetra-*n*-butylammonium iodide (TBAI), tetrahydrofuran/*N*,*N*-dimethylformamide (THF/DMF; 1:1), 0°C to RT, 2.5 h, 83%; l) 80% AcOH, 80°C, 24 h; m) Ac_2O, DMAP, pyridine, 0°C to RT, 15 h, 95% over two steps; n) thymine, *N*,*O*-bis(trimethylsilyl)acetamide (BSA), 1,2-dichloroethane, 80°C, 1 h; then trimethylsilyl trifluoromethanesulfonate (TMSOTf), 0°C to 50°C, 19 h, 84%; o) K_2CO_3, MeOH, RT, 15 h; p) methanesulfonyl chloride (MsCl), pyridine, 0°C to RT, 12 h, 86% over two steps. q) DBU, MeCN, 80°C, 12 h; r) NaOH, EtOH/H_2O (1:1), 90°C, 2 h; s) DBU, BOMCl, DMF, 0°C, 1 h, 65% over three steps; t) Tf_2O, pyridine, CH_2Cl_2, 0°C, 30 min.; u) $NaNH_2$, DMF, 55°C, 15 min., 52% over two steps; v) 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), CH_2Cl_2/H_2O (9:1), RT, 1 h, 92%; w) levulinic acid (LevOH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl), iPr_2NEt, DMAP, CH_2Cl_2, RT, 10 h, 84%; x) H_2, $Pd(OH)_2$/C, MeOH/EtOAc (1:1), RT, 3 d; then iPr_2NEt, RT, 1 h, 95%; y) $NC(CH_2)_2OP(NiPr_2)_2$, 1*H*-tetrazole, *N*-methylimidazole, DMF, RT, 6 h, 55%.

anhydronucleoside intermediate **18**, followed by basic hydrolysis, and benzyl chloromethyl ether (BOM) protection of the imide nitrogen atom gave the C2′-inverted nucleoside **19**. Conversion of **19** to the corresponding triflate gave **20**, which upon exposure to $NaNH_2$ in *N*,*N*-dimethylformamide (DMF) gave **21**, containing the tricyclic core of **9**, in 52% yield over two steps. Cleavage of the *O*-Nap group, conversion to the levulinate ester, and hydrogenolysis, followed by phosphitylation led to **23**, the crucial monomer in the synthesis of the oligonucleotides for melting temperature (T_m) studies.

Confirmation of the structure of the tricyclic spironucleoside **21** was found through an X-ray crystal structure of a corresponding *p*-nitrobenzoate ester.[14] Considering the possibility for β-elimination of the triflate ester to occur in **20**, it is remarkable that intramolecular S_N2 displacement prevailed to give **21** as the major product, even on gram scale. Nevertheless, the formation of tricyclic nucleoside **21** was accompanied by a benzyl enol ether by-product, likely resulting from triflyl migration to the 2′-OH group and subsequent elimination.[14]

We measured the ability of tricyclic nucleoside motif **9** to stabilize oligonucleotide duplexes versus complementary DNA and RNA using two oligonucleotide sequences (Table 1). Oligonucleotide syntheses were carried out on 1 μmol scale with universal UnyLinker polystyrene support. Standard conditions were used to incorporate the deoxynucleotides, and **23** was incorporated by manual coupling followed by removal of the levulinyl protecting group using hydrazine in pyridine/acetic acid.[14,15] The tricyclic analogue **9** (**A4**, ΔT_m +7.1 °C/modification) showed excellent duplex-stabilizing properties versus complementary RNA relative to LNA (**A2**, ΔT_m +5.2 °C/mod.) and α-L-LNA (**A3**, ΔT_m +5.7 °C/mod.) when inserted in a stretch of dT residues. Analogue **9** (**A4**, ΔT_m +2.6 °C/mod.) also showed good duplex stabilization versus complementary DNA as compared to LNA (**A2**, ΔT_m +1.7 °C/mod.) and α-L-LNA (**A3**, ΔT_m +1.4 °C/mod.), although the magnitude of stabilization was smaller given the higher baseline T_m of the parent sequence **A1** for its DNA complement.[16] In addition, **9** showed excellent mismatch discrimination properties, which were comparable or improved relative to DNA, LNA, and α-L-LNA modifications at the same position.[14] The tricyclic analogue **9** also showed excellent duplex-stabilizing properties versus RNA and DNA complements in a second sequence (**A7**, **A9**, and **A11**, ΔT_m +4.7 to +8.3 °C/mod. for RNA and +3.0 to +7.4 °C/mod. for DNA) as compared to α-L-LNA (**A6**, **A8**, and **A10**, ΔT_m +4.5 to +6.3 °C/mod. for RNA and +3.8 to +6.5 °C/mod. for DNA).

In conclusion, we show that a strategy of dual conformational restriction can indeed be useful for stabilizing oligonucleotide duplexes. The duplex-stabilizing properties of **9** are impressive because several previous attempts to increase duplex thermostability by appending six-membered rings to restrict conformational freedom of the nucleoside furanose ring were unsuccessful.[17] It is conceivable that the duplex-stabilizing properties of **9** could be further improved by introducing heteroatoms or other polar functional groups to the six-membered carbocyclic ring to preserve the water of hydration network around the sugar–phosphate backbone.[18] Further attempts to apply this strategy of dual conformational restriction to other classes of nucleic acid analogues, and to investigate its potential for the antisense approach, are currently in progress and the results of these experiments will be reported later.

Table 1: Duplex thermal-stability measurements of LNA-, α-L-LNA-, and 2′,4′-5′,6′-bc-α-L-TriNA-modified oligonucleotides versus DNA and RNA complements.[a]

No.	Modification	Sequence (5′- 3′)	ΔT_m °C vs. DNA	ΔT_m °C vs. RNA
A1	DNA	d(GCGTTTTTTGCG)	(49.1)	(46.0)
A2	LNA	d(GCGTT**T**TTTGCG)	+1.7	+5.2
A3	α-L-LNA	d(GCGTT**T**TTTGCG)	+1.4	+5.7
A4	**9**	d(GCGTT**T**TTTGCG)	+2.6	+7.1
A5	DNA	d(CCAGTGATATGC)	(47.3)	(43.6)
A6	α-L-LNA	d(CCAG**T**GATATGC)	+3.8	+5.6
A7	**9**	d(CCAG**T**GATATGC)	+3.0	+5.3
A8	α-L-LNA	d(CCAGTGA**T**ATGC)	+6.5	+6.3
A9	**9**	d(CCAGTGA**T**ATGC)	+7.4	+8.3
A10	α-L-LNA	d(CCAGTGATA**T**GC)	+4.4	+4.5
A11	**9**	d(CCAGTGATA**T**GC)	+4.4	+4.7

[a] T_m values were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA using complementary RNA 5′-r(AGCAAAAAACGC)-3′ or DNA 5′-d(AGCAAAAAACGC)-3′ for **A1**–**A4** and RNA 5′-r(GCAUAUCACUGG)-3′ or DNA 5′-d(GCATATCACTGG)-3′ for **A5**–**A11**, where **T** indicates the site of modification. Final duplex concentration was 4 μM. T_m values reflect the average of three measurements (standard deviation ±0.02–0.18 °C).

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[1] E. E. Swayze, B. Bhat in *Antisense Drug Technology: Principles, Strategies, and Applications*, 2nd ed. (Ed.: S. T. Crooke), CRC, Boca Raton, **2008**, pp. 143–182.
[2] M. E. Østergaard, P. J. Hrdlicka, *Chem. Soc. Rev.* **2011**, *40*, 5771–5788.
[3] a) B. Vester, J. Wengel, *Biochemistry* **2004**, *43*, 13233–13241; b) C. J. Leumann, *Bioorg. Med. Chem.* **2002**, *10*, 841–854; c) P. Herdewijn, *Chem. Biodiversity* **2010**, *7*, 1–59; d) A. Eschenmoser, *Pure Appl. Chem.* **1993**, *65*, 1179–1188.
[4] D. Renneberg, C. J. Leumann, *J. Am. Chem. Soc.* **2002**, *124*, 5993–6002.
[5] C. Dupouy, N. Iche-Tarrat, M. P. Durrieu, F. Rodriguez, J. M. Escudier, A. Vigroux, *Angew. Chem.* **2006**, *118*, 3705–3709; *Angew. Chem. Int. Ed.* **2006**, *45*, 3623–3627.
[6] a) J. Wengel, *Acc. Chem. Res.* **1999**, *32*, 301–310; b) T. Imanishi, S. Obika, *Chem. Commun.* **2002**, 1653–1659.
[7] a) V. K. Rajwanshi, A. E. Håkansson, M. D. Sørensen, S. Pitsch, S. K. Singh, R. Kumar, P. Nielsen, J. Wengel, *Angew. Chem.* **2000**, *112*, 1722–1725; *Angew. Chem. Int. Ed.* **2000**, *39*, 1656–1659; b) M. D. Sørensen, L. Kværnø, T. Bryld, A. E. Håkansson, B. Verbeure, G. Gaubert, P. Herdewijn, J. Wengel, *J. Am. Chem. Soc.* **2002**, *124*, 2164–2176.

[8] M. Petersen, A. E. Håkansson, J. Wengel, J. P. Jacobsen, *J. Am. Chem. Soc.* **2001**, *123*, 7431 – 7432.
[9] J. T. Nielsen, P. C. Stein, M. Petersen, *Nucleic Acids Res.* **2003**, *31*, 5858 – 5867.
[10] a) P. P. Seth, J. Yu, C. R. Allerson, A. Berdeja, E. E. Swayze, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 1122 – 1125; b) P. P. Seth, C. A. Allerson, M. E. Østergaard, E. E. Swayze, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 296 – 299.
[11] J. K. Maity, R. Ghosh, M. G. B. Drew, B. Achari, S. B. Mandal, *J. Org. Chem.* **2008**, *73*, 4305 – 4308.
[12] a) A. Hosomi, M. Endo, H. Sakurai, *Chem. Lett.* **1976**, 941 – 942; b) A. Hosomi, H. Sakurai, *Tetrahedron Lett.* **1976**, *17*, 1295 – 1298; c) S. J. Danishefsky, M. DeNinno, *Tetrahedron Lett.* **1985**, *26*, 823 – 824.
[13] U. Niedballa, H. Vorbrüggen, *Angew. Chem.* **1970**, *82*, 449 – 450; *Angew. Chem. Int. Ed. Engl.* **1970**, *9*, 461 – 462.
[14] See Supporting Information for details.
[15] A. Romieu, D. Gasparutto, J. Cadet, *J. Chem. Soc., Perkin Trans. 1* **1999**, 1257 – 1264.
[16] S. M. Abdur Rahman, S. Seki, S. Obika, H. Yoshikawa, K. Miyashita, T. Imanishi, *J. Am. Chem. Soc.* **2008**, *130*, 4886 – 4896.
[17] a) K. I. Shaikh, S. Kumar, L. Lundhus, A. D. Bond, P. K. Sharma, P. Nielsen, *J. Org. Chem.* **2009**, *74*, 1557 – 1566; b) A. Stauffiger, C. J. Leumann, *Eur. J. Org. Chem.* **2009**, 1153 – 1162; c) M. Sekiguchi, S. Obika, Y. Harada, T. Osaki, R. Somjing, Y. Mitsuoka, N. Shibata, M. Masaki, T. Imanishi, *J. Org. Chem.* **2006**, *71*, 1306 – 1316; d) J. Ravn, N. Thorup, P. Nielsen, *J. Chem. Soc., Perkin Trans. 1* **2001**, 1855 – 1861.
[18] a) S. Kumar, M. H. Hansen, N. Albaek, S. I. Steffansen, M. Petersen, P. Nielsen, *J. Org. Chem.* **2009**, *74*, 6756 – 6769; b) P. P. Seth, C. R. Allerson, A. Berdeja, A. Siwkowski, P. S. Pallan, H. Gaus, T. P. Prakash, A. T. Watt, M. Egli, E. E. Swayze, *J. Am. Chem. Soc.* **2010**, *132*, 14942 – 14950.

Communications

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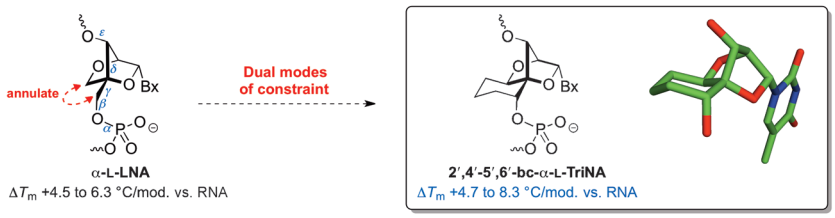
Structure-Based Design of a Highly Constrained Nucleic Acid Analogue: Improved Duplex Stabilization by Restricting Sugar Pucker and Torsion Angle γ



Dual conformational restriction: A new, highly constrained modification of the α-L-locked nucleic acid (α-L-LNA) scaffold that locks the sugar furanose ring in an N-type configuration and also restricts rotation around torsion angle γ was synthesized (see scheme). This new modification increases the thermostability of an oligonucleotide duplex compared to using a single mode of constraint alone.