Pergamon

Tetrahedron Letters, Vol. 35, No. 11, pp. 1647-1650, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(94)E0150-V

Synthesis of a Disulfide Stabilized RNA Hairpin[1]

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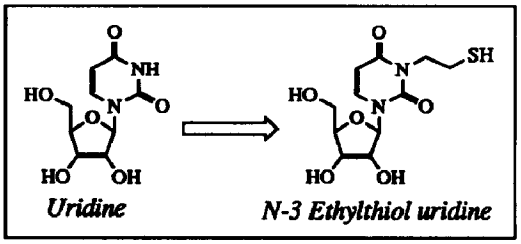
Key Words: Disulfide cross-link, RNA hairpin

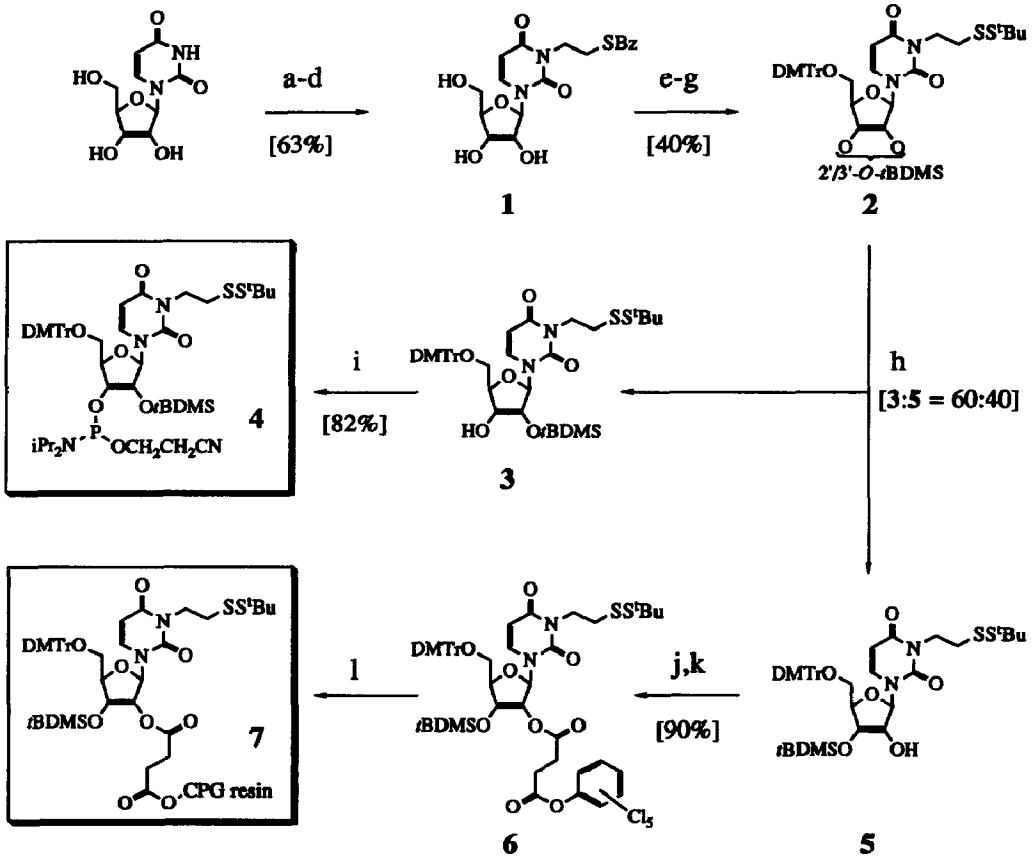
Abstract: **An *N*-3 ethylthiol-modified uridine has been synthesized and incorporated through solid-phase phosphoramidite chemistry at the 5'- and 3'-termini of an RNA hairpin to provide increased conformational stability via a disulfide cross-link.**



The remarkable conformational diversity of RNA is manifested in the variety of its biological functions, such as in transfer, messenger and ribosomal RNA, and ribozymes. Hairpin loops contribute significantly to these structures and have been implicated as sites for protein binding and nucleation of RNA folding.[3,4] In particular, tetranucleotide loop sequences U*N*CG and G*NR*A (where *N* is any nucleotide and *R* is purine) frequently occur in RNA hairpins and confer extraordinary stability to these structures.[5,6,7] The UUCG tetraloop is found frequently within the mRNA of bacteriophage T4,[5] and has been speculated to be a potential protein binding site for a variety of systems.[5] To facilitate examination of the roles of hairpin loops and duplex major groove accessibility to protein recognition and binding,[8,9] we have incorporated a disulfide cross-link at the terminus of an RNA hairpin using an *N*-3 alkylthiol-modified uridine and demonstrate that this cross-link imparts added conformational stability.[10,11] Cross-linking at the terminus does not disrupt Watson-Crick base-pairing,[12] and unlike cross-links positioned within the duplex[13] does not potentially interfere with protein binding and hydration in the major groove.

The *N*-3 ethylthiol-modified uridine was synthesized by alkylation of transiently protected uridine[14] to provide **1** in 63% yield (SCHEME I). Following tritylation[15] of the 5'-hydroxyl group the thiobenzoate was converted to the *t*-butyl disulfide,[16,17] with subsequent monosilylation[15] to yield a mixture of 2'- and 3'-*O*-silylated intermediates **2** in 40% yield for three steps. The 2'- and 3'-isomers were separated by flash

chromatography[18] and the 2'-silyl ether **3** was converted to the 3'-phosphoramidite[19] **4** in 82% yield.[20] To incorporate the modified uridine at the 3'-terminus of an RNA oligomer requires preparation of the appropriate nucleoside-modified resin. This support was synthesized by treatment of the 3'-silyl ether **5** with succinic anhydride and activation of the free carboxylate as the pentachlorophenyl ester, **6**.[21] Reaction of aminoalkylated controlled-pore glass (500 Å) with **6** provided the desired resin, **7**, with a loading of 30 μmol/g.



SCHEME I. (a) TMSCl, Et_3N, DMF; (b) NaH, DMF; (c) pTsOCH$_2$CH$_2$SBz; (d) HF(aq.); (e) DMTrCl, pyridine; (f) 1-*t*-butylthiohydrazine-1,2-dicarboxmorpholide, LiOH, MeOH; (g) *t*-BDMSCl, imidazole, DMF; (h) flash chromatography; (i) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, 2,4,6-collidine, *n*-methylimidazole, THF; (j) succinic anhydride, DMAP, pyridine; (k) pentachlorophenol, DCC, DMAP, $MeCl_2$; (l) CPG resin, Et_3N, DMF.

The RNA sequence 5'-𝕌GACUUCGGUCU (𝕌 = *N*-3 ethyl-*t*-butyldisulfide uridine) was chosen due to the availability of thermodynamic and NMR structural data for the parent UUCG RNA tetraloop hairpin (5'-GGACUUCGGUCC).[7] Previous crystallization of this sequence resulted in a bulged duplex due to the requisite high salt and RNA concentrations utilized.[22] Conformational homogeneity enforced by the disulfide cross-link will potentially address this problem for structure determination. The RNA was synthesized via solid-phase phosphoramidite chemistry on a Millipore Expedite synthesizer, followed by cleavage of the oligomer from the resin with anhydrous ethanolic ammonia at room temperature and removal of the base and phosphodiester protecting groups at 55 °C for 12 h. Treatment of the base-deprotected oligomer with tetrabutylammonium fluoride (1 M in THF) at room temperature for 18 h to remove the 2'-silyl groups and desalting on a Qiagen 500 cartridge yielded the fully deprotected *t*-butyl disulfide RNA hairpin. Purification from failed sequences was achieved by anion-exchange HPLC with a Partisphere SAX column, eluting with a gradient of 15 mM to 300 mM KH_2PO_4 (pH 6.3) containing 20% CH_3CN. Subsequent desalting was performed with a Waters C-18 Sep-Pak, eluting with a CH_3CN step gradient, obtaining the pure RNA hairpin in roughly 45% overall yield (based on a 1 μmole scale synthesis).[23,24]

The *t*-butyl thiol protecting groups were removed by treatment of the *t*-butyl disulfide RNA hairpin with dithiothreitol (100 equivalents) at pH 8, 40 °C for 12 h (monitored by reversed-phase HPLC on a Vydac C-4 column, eluting with a CH_3CN gradient in 0.1 M Et_3NHOAc, pH 6.6). This deprotection was followed by Qiagen cartridge purification which yielded the alkylthiol-modified RNA hairpin. Air oxidation of this material (5 hours, pH 8) under conditions which favor hairpin formation (100 mM NaH_2PO_4, 1 mM in RNA) provided the disulfide cross-linked hairpin as verified by a negative Ellman's test and native and denaturing polyacrylamide gel electrophoresis.[10a,b] Enhanced stability due to the disulfide is indicated by comparison of the UV thermal denaturation curves for the cross-linked and unmodified RNA hairpins; T_m for the disulfide cross-linked hairpin is 90 °C (10 mM NaH_2PO_4, pH 6.0), almost 20 °C greater than for the unmodified hairpin (72 °C).[7,25] Notably, the T_m for the tetraloop hairpin with the terminal *t*-butyl disulfide modified uridines is virtually identical with that for the wild type sequence, suggesting that the terminus of the RNA helix can accommodate a remarkable degree of steric bulk without adversely affecting its stability.[10b,12]

In conclusion we have synthesized an *N*-3 ethylthiol-modified uridine and incorporated it at both the 5'- and 3'-termini of an RNA hairpin to provide a disulfide cross-link. We have demonstrated that this cross-link

imparts greater conformational stability to the hairpin, and as such will be useful in the study of protein-RNA and RNA-RNA recognition. Insuring conformational homogeneity for such structures should also assist greatly in NMR and crystallographic studies.[6,7,22]

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(*Received in USA* 16 *November* 1993; *revised* 22 *December* 1993; *accepted* 11 *January* 1994)