Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lsyc20

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Published online: 04 Dec 2007.

To cite this article: Jasenka Matulic-Adamic & Leonid Beigelman (2000) An Improved Synthesis of Inosine 3′-Phosphoramidite, Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry, 30:21, 3963-3969

To link to this article: http://dx.doi.org/10.1080/00397910008086956

SYNTHETIC COMMUNICATIONS, 30(21), 3963–3969 (2000)

AN IMPROVED SYNTHESIS OF INOSINE 3′-PHOSPHORAMIDITE

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Abstract: A protocol for the efficient conversion of inosine into its 3′-phosphoramidite synthon for solid phase oligonucleotide synthesis has been developed.

There are a number of examples where guanosine-uridine (G-U) pairs play critical roles in RNA function. For example, a phylogenetically conserved G-U pair defines the 5′-exon/intron boundary in the group I self-splicing intron[1] and the G3-U70 base pair was identified as a major determinant of the identity of an alanine tRNA.[2] Conserved G-U pairs are also found within domain 5 of the group II intron[3] and at the cleavage site of the HDV ribozyme.[4]

Inosine is an analog of guanosine that lacks the 2′-amino group of the guanine base. It forms a wobble-like base pair with uridine (I-U) that retains all major and minor groove base atoms except for the 2-amino group present in the G-U wobble pair. Because of the similarity of I-U and G-U pairs, inosine substitution has been used to elucidate various structural and/or functional components of a number of RNAs. Inosine substitution was used to determine the recognition elements of the G-U wobble in the case of the *Tetrahymena* intron.[5] A similar approach was used to reveal that an unpaired guanine 2-amino group in the minor groove of the RNA helix marks the Ala tRNA for aminoacylation with alanine.[6] In addition, inosine substitutions were used to study the importance of functional groups at conserved purine positions in the catalytic core of the hammerhead ribozyme[7-9] and to support the hypothesis that the guanine 2-amino group is directly involved in the catalytic mechanism of the hairpin ribozyme.[10]

Inosine modification of the highly conserved G3 residue within Domain 5 of the Group II Intron was instrumental in the elucidation of the dual functionality (minor groove binding and major grove catalysis) of this catalytically essential residue.[11] Finally, it was recently discovered that an adenosine to inosine mutation at position 15.1 of the hammerhead domain changes the substrate "cleavage rule" of the hammerhead ribozyme from NUH to NCH, thus allowing for the cleavage of additional sites.[12]

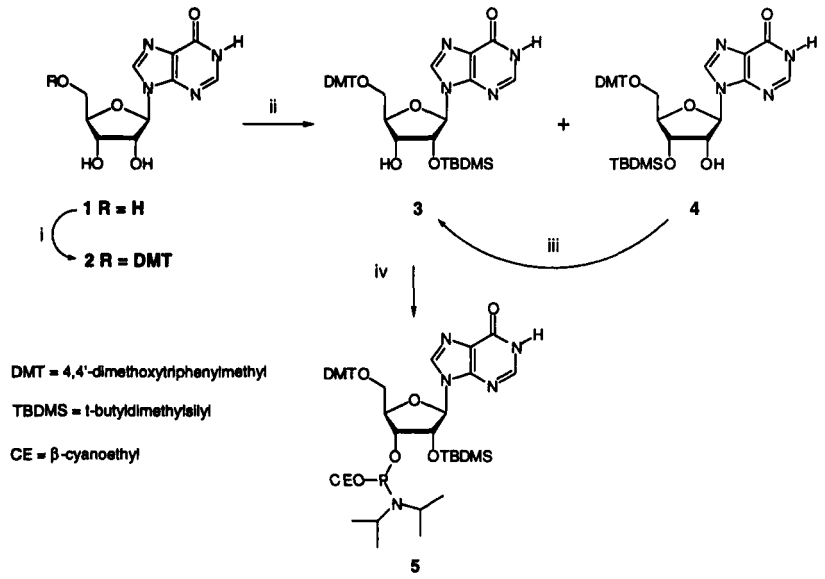
The increasing role of inosine in biological studies prompted us to search for an improved synthesis of inosine phosphoramidite **5**, a building block for the site-specific incorporation of riboinosine residues into oligonucleotides by solid phase phosphoramidite method. Previously, Kawase *et al.* described preparation of inosine 3'-*O*-phosphoramidite synthone with 2'-OH protected by tetrahydropyranyl group.[13] Charubala and Pfleiderer[14] reported preparation of 5'-*O*-momethoxytrityl 2'(3')-*O*-*t*-butyldimethylsilyl inosine intermediate in low yield which was used for the synthesis of 2'-5' IpIpI analog of 2'-5' ApApA by phosphate triester method. Finally, Green *et al.*[6] reported the synthesis of **5** in 24% overall yield from **1**. Our procedure provides **5** in 54% overall yield from **1**. It was also optimized for the scale-up to multigram quantities.

Currently, standard protecting groups for the solid phase oligoribonucleotide synthesis are the dimethoxytrityl ether for the 5'-hydroxyl group[15] and the *t*-butyldimethylsilyl ether for the 2'-hydroxyl group.[16] The low solubility of inosine derivatives in common organic solvents makes selective introduction of these protecting groups and subsequent 3'-*O*-phosphitylation quite challenging.

We found that highly selective 5'-*O*-tritylation of **1** can be achieved by portionwise addition of DMT-chloride. Adding the reagent at once led to the formation of substantial quantities of 2',5'- and 3',5'-bis-*O*- substituted derivatives as judged by TLC. Also, the addition of DMAP and triethylamine as catalysts increased the formation of byproducts.

Silylation of **2** using the procedure of Hakimelahi *et al*[17] resulted, as judged by TLC, in selective protection of the 2'-hydroxyl to give **3**. This derivative crystallized cleanly from ethyl acetate in 45% yield. The mother liquor was enriched for the 2'-isomer **3** by treatment with 3% triethylamine in methanol yielding a 1:1 ratio of isomers **3** and **4**. Additional quantity of 2'-isomer crystallized from this mixture resulting in 76% overall yield of **3**. To the best of our knowledge this is the first example where 2' and 3'-*O*-silyl isomers of 5'-*O*-DMT ribonucleosides are cleanly separated by crystallization, eliminating the need for tedious separation by column chromatography.

Synthesis of Inosine 3'-phosphoramidite



Reagents and conditions: **(i)** DMTCl/Py/DMSO, rt, 48 h; **(ii)** TBDMSiCl/$AgNO_3$/Py/THF, overnight; **(iii)** 3% Et_3N/MeOH, 3 h; **(iv)** 2-cyanoethyl N,N-diisopropylchlorophosphoramidite/1-MeIm/DIPEA/DCM, rt, 2 h.

In the reported[6] phosphitylation of **3**, an excess of collidine was used to solubilize the protected nucleoside that is sparsely soluble in the solvents used in standard phosphitylation reactions. In our hands collidine often interferes with both TLC and column chromatographic separations and it is difficult to completely remove it by evaporation under reduced pressure. Our procedure uses *N,N*-diispropylethylamine as a base. We have tried many TLC solvents to follow the progress of the phosphitylation reaction but all of them failed to separate starting material from the product. In addition, ^{31}P and ^{1}H NMR proved to be unreliable in monitoring the progress of the phosphytilation; therefore, an excess of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite and 1-methylimidazole was used in order to insure that the reaction goes to completion. Avoiding the aqueous work-up eliminated the formation of the undesired

hydrogenphosphonate byproducts allowing for the high purity of the final product **5**.

NMR spectra of **2**, **3**, **4** and **5** were in accordance with the published data.[6] We have incorporated monomer **5** in several modified oligoribonucleotides by standard procedure[18] and confirmed structure of these oligonucleotides by ES/MS and base compositional analysis.[11] We found that coupling efficiency of **5**, assayed by measuring the trityl cation released, was >98%.

In conclusion, we have developed an improved and reliable procedure for the synthesis of inosine phosphoramidite with minimal number of the column purification steps.

Experimental

General. All reactions were carried out under a positive pressure of argon in anhydrous solvents. Commercially available reagents and anhydrous solvents were used without further purification. NMR spectra were recorded on a Varian Gemini 400 spectrometer operating at 400. 75 MHz for ^{1}H and 161.947 MHz for ^{31}P with tetramethylsilane and 85% phosphoric acid as external standard respectively. Analytical thin-layer chromatography (TLC) was performed with Merck Art. 5554 Kieselgel 60 F_{254} plates and flash column chromatography using Merck 0.040-0.063 mm silica gel 60. Melting temperatures were determined on the Electrothermal Model IA 9200 apparatus and are uncorrected. Elemental analyses were performed by MHW Laboratories, Phoenix, AZ.

5'-*O*-Dimethoxytrityl inosine (2). Inosine (**1**) (10 g, 37.3 mmol) is dried overnight under high vacuum at 100 °C, then dissolved in dry pyridine-DMSO 1:1 mixture (85 ml) under argon. 4,4'-Dimethoxytrityl chloride (15.17 g, 44.8 mmol, 1.2 eq) is added in 8 hourly portions[19] and the reaction mixture is then stirred under argon at rt. overnight. Methanol (20 ml) is added, the solution stirred for 15 min and partitioned between dichloromethane (300 ml) and saturated aqueous $NaHCO_3$ (300 ml). The aqueous layer is extracted with dichloromethane (300 ml), the organic layers are combined, washed with brine (300 ml) and dried (Na_2SO_4). Solvents are removed *in vacuo* and the residue coevaporated twice with toluene to remove residual pyridine and DMSO.[20] Flash silica gel column chromatography using 1-20% gradient of methanol in dichloromethane cleanly removes the bis-substituted products and unreacted starting material to afford product as a white foam (18.3 g,

86%), Anal. Calcd for $C_{31}H_{30}N_4O_7$: C, 65.25; H, 5.30 ; N, 9.82. Found: C, 65.13; H, 5.20; N, 9.88.

5'-*O*-Dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl inosine (3). 5'-*O*-Dimethoxytrityl inosine (2) (18 g, 31.55 mmol) is dissolved in dry THF (200 ml) and dry pyridine (20 ml) is added under argon. $AgNO_3$, finely powdered and dried under vacuum at 100 ^{0}C overnight (6.97 g, 41 mmol, 1.3 eq) is added and the mixture stirred until solid dissolves (ca 15 min). *t*-Butyldimethylsilyl chloride (6.18 g, 41 mmol, 1.3 eq) is then added and the mixture stirred at rt. overnight. An additional 1.6 g, (0.3 eq) of $AgNO_3$ and 1.4 g, 0.3 eq of TBDMS-Cl is added and the mixture is stirred an additional 24 hours.[21] The mixture is filtered through the Celite pad into saturated aqueous $NaHCO_3$ solution (300 ml) and the filtrate extracted with 2 x 300 ml dichloromethane. Organic layers are combined, washed with brine (300 ml), dried (Na_2SO_4) and concentrated to a syrup *in vacuo*. The residue (insoluble in dichloromethane or chloroform) is dissolved in boiling ethyl acetate (800 ml), the white precipitate which forms on standing at rt. overnight is filtered off and dried (9.7 g, 45%). The mother liquor which contains mono and bis substituted derivatives is concentrated *in vacuo* and then dissolved in 3% triethylamine in methanol (200 ml). After 3 hours at rt. solvents are removed and the residue crystallized as above to afford additional 6.8 g (31%) of the product. Total yield: 16.5 g, 76%, m.p. 219-220 ^{o}C, Anal. Calcd for $C_{37}H_{44}N_4O_7Si$: C, 64.89; H, 6.48; N, 8.18. Found: C, 64.90; H, 6.23; N, 8.28.

5'-*O*-Dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl inosine 3'-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (5). To a stirred suspension of 5'-*O*-dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl inosine (3) (5 g, 7.30 mmol) in dry dichloromethane (85 ml) under argon, *N,N*-diisopropylethylamine (6.36 ml, 36.5 mmol, 5 eq) and 1-methylimidazole (0.29 ml, 3.65 mmol, 0.5 eq) are added followed immediately by 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (4.07 ml, 18.25 mmol, 2.5 eq). A clear solution results and stirring is continued for 3 hours at rt. In all TLC solvents tried the product is indistinguishable from starting material (both have the Rf *ca* 0.15 in acetone-ethyl acetate-triethylamine 60:30:10). Smearing is seen in all solvents. The reaction mixture is cooled to 0 ^{o}C (ice bath), dry ethanol (10 ml) is added and the solution stirred for 15 min at 0 ^{o}C. It is then pump-loaded on the short column of silica gel equilibrated with 5% triethylamine/hexanes. Elution is carried out with hexanes-ethyl acetate 1:1 to elute the reagents followed by ethyl acetate, ethyl acetate-

acetone 1:1 and finally acetone. All solvents contained 5% TEA. The product is obtained as a white foam after removal of solvents (5.33 g, 83%). Finally, the material is dissolved in acetonitrile and filtered through a 0.45 μm PTFE filter (Millipore), dried under high vacuum and stored under argon at –20 °C.

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19. Addition of DMT-Cl in 6 portions in 30 min. intervals resulted in a similar yield of the final product.
20. Product can be crystallized from isopropanol at this stage but the recovery is lower then 50%.

21. It is important that little or no starting material remains at the end of reaction because it can co-crystallize with the product from ethyl acetate.

Received in the USA 2/13/00