Facile synthesis of a 3-deazaadenosine phosphoramidite for RNA solid-phase synthesis

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Abstract
Access to 3-deazaadenosine (c₃A) building blocks for RNA solid-phase synthesis represents a severe bottleneck in modern RNA research, in particular for studies that aim at the mechanistic elucidation of site-specific backbone cleavage of recently discovered ribozyme classes, known as twister, twister sister, pistol, and hatchet RNA motives [1,2]. Selected adenines in their active sites have been discussed to participate in acid base catalysis, thereby contributing to accelerate the specific phosphodiester cleavage of these nucleolytic ribozymes. Concerning the twister ribozyme, structural analyses suggest that an adenine N3 atom plays a dominant role in catalysis [3-5]. Also for the pistol ribozyme, evidence exists that an adenine-N3 in the active site is significant for the cleavage activity, most likely by 5’-O-leaving group stabilization through proton shuttling [6,7]. Another example for a specific role of an adenine-N3 is associated with the catalysis during ribosomal peptide bond formation, a proposal about its role in proton transfer has been disputed heavily since the first ribosome crystal structures up to very recent investigations [8-10]. The involvement of N3, and not N1, is surprising with respect to basicity of these purine nitrogen atoms, because N1 represents the major protonation site, followed by N7 and N3. This order is deduced from the macroscopic pKₐ values that were measured for adenine, 9-methyladenine, and adenosine [11]. Importantly, there is growing evidence that the pKₐ values of nucleobases can be significantly shifted within a well-structured RNA fold [12-15].
To address RNA phenomena of that kind, comparative atomic mutagenesis is an indispensable means, and with respect to ribozymes, can deliver important insights into the RNA catalyzed chemical reactions and underlying mechanisms. Therefore, 1-deazaadenosine (c3A), 1-deaza-2'-deoxyadenosine (c3dA), 3-deazaadenosine (c3A), and 3-deaza-2'-deoxyadenosine (c3dA), and the corresponding phosphoramidites to prepare oligoribonucleotides are highly requested nucleoside modifications. Unfortunately, synthetic approaches to achieve them are troublesome and time consuming, in particular for c3A. To the best of our knowledge, only two papers have reported the synthesis of c3A phosphoramidites so far [16,17]. Thereby, the major bottleneck is access to the naked nucleoside. Although the c3A nucleoside is commercially available, prices in the hundreds of Euro range for low milligram amounts make this source unsatisfying. The previously reported c3A phosphoramidite synthesis from our laboratory [16], which took older reports by Matsuda, Piccialli, McLaughlin, Watanabe, Robins, and co-workers into account [17-21], started from inosine leading to c3A after 8 steps via a 5-amino-4-imidazolecarboxamide (AICA) riboside derivative with 8% overall yield. Another 4 steps followed to achieve a properly protected building block for RNA solid-phase synthesis [16]. With a total of 12 steps, the approach is not very attractive. Because of this frustrating situation, we set out to develop an efficient and easy-to-handle synthesis of a 3-deazaadenosine phosphoramidite building block.

**Results and Discussion**

**Previously described synthetic routes to c3A via nucleosidation**

In 1966, Rousseau, Townsend, and Robins reported the nucleosidation of 4-chloroimidazo[4,5-c]pyridine and 1,2,3,5-tetraacetyl-ß-D-ribofuranose in the presence of chloro acetic acid to yield the corresponding 6-chloro-3-deazapurine nucleoside (Scheme 1) [22]. Subsequent attempts to convert the chlorine atom directly by amination under various conditions failed. Only when treated with hydrazine, nucleophilic substitution was observed and after reduction with Raney nickel the desired 3-deazaadenosine was isolated. Our own attempts towards direct ammonolysis failed as well. Additionally, the limited commercial availability of hydrazine and its inconvenience in handling excluded this route for our purposes.

In 1977, Montgomery, Shortnacy, and Clayton, reported the preparation of 6-chloro-3-deazapurine ribonucleoside via nucleosidation of 4,6-dichlorimidazo[4,5-c]pyridine with 1,2,3,5-tetraacetyl-ß-D-ribofuranose in the presence of p-toluenesulfonic acid (Scheme 2) [23,24]. Treatment of the 2,6-dichloro-3-deazapurine derivative with ammonia was opti-

### Scheme 1: Synthesis of c3A described by Rousseau et al. in 1966 [22].

| Step | Reaction Conditions |
|------|---------------------|
| a    | 1,2,3,5-Tetraacetyl-D-ribofuranose, chloroacetic acid (cat.), 175 °C (melting until clear solution). |
| b    | NH3 in CH3OH, 0 °C, 14 h. |
| c    | Anhydrous hydrazine, steam bath, 1 h, not isolated. |
| d    | Raney nickel, water, reflux, 1 h. |

### Scheme 2: Synthesis of c3A described by Montgomery et al. in 1977 [23].

| Step | Reaction Conditions |
|------|---------------------|
| a    | 1,2,3,5-Tetraacetyl-D-ribofuranose, chloroacetic acid (cat.), melt (160 °C), 5 to 10 min. |
| b    | NH3 in ethanol (saturated at ~30 °C), 140 °C, 89 h [23] or NH3 (30% aq), 200 °C, 5 d, 80% [24]. |
mized by Bande et al. recently [25], but still required 200 °C reaction temperature and five days reaction time to afford regio-
selective displacement of the 2-chlorine atom and concomitant deacetylation in high yield. Unfortunately, all attempts of the
authors to displace the second chlorine atom of the imidazo[4,5-
c]pyridine nucleoside using sodium methoxide or palladium-
catalyzed cross-coupling reactions as described in [26] failed.
We therefore decided not to put additional efforts into this
route.

**Attempts to use 6-azido-3-deazapurine ribonucleoside as key intermediate**

Our initial attempts to create an efficient route to c^3^A started
with the smooth transformation of commercially available
4-chloroimidazo[4,5-c]pyridine with lithium azide to provide
4-azidoimidazo[4,5-c]pyridine (1) [27] (Scheme 3). Then,
glycosylation with 1-O-acetyl-2,3,5-tri-O-benzoyl-ß-D-ribofu-
ranose gave the desired nucleoside 2 in high yield. Unfortu-
nately, all our attempts to find appropriate conditions to reduce
the 6-azido group to the corresponding amine failed. In short,
these trials included i) hydrogenation under Pd/C catalysis at
 elevated pressure (30 psi) in ethanol or N,N-dimethylacetamide,
ii) ammonium formiate, Pd/C, in methanol [28], iii) tin(II) chlo-
ride, in ethanol [29], iv) thioacetic acid, lutidine, in CH_2Cl_2
[30], v) triphenylphosphine, in CH_2Cl_2, aqueous work-up, and
finally vi) Mg_0 in methanol.

**Efficient 5-step synthesis of 3-deazaadeno-
sine phosphoramidite**
The key step of our novel route to c^3^A phosphoramidite
(Scheme 4) is a silyl-Hilbert–Johnson nucleosidation reaction of
commercially available 4-aminoimidazo[4,5-c]pyridine (3) and
1-O-acetyl-2,3,5-tri-O-benzoyl-ß-D-ribofuranose in the pres-
ence of N,O-bis(trimethylsilyl)acetamide and trimethylsilyl tri-
fluoromethanesulfonate in toluene. No protection of the
4-amino group of compound 3 was required. The reaction
proceeded in high yields and gave the tribenzoylated c^3^A
nucleoside 4. This compound was analysed by 1^H ROESY

**Scheme 3**

**Scheme 4**

Efficient 5-step synthesis of 3-deazaadenosine phosphor-
amidite 8 from commercially available, affordable starting materials.
(A) Reagents and conditions: a) N,O-bis(trimethylsilyl)acetamide
(3.5 equiv), trimethylsilyl trifluoromethanesulfonate (3.0 equiv), in tolu-
ene, 105 °C, 3.5 h, 63%. b) CH_3NH_2, in water/ethanol, room tempera-
ture, 18 h, 95%. c) i) N,N-Dibutylformamide dimethyl acetal (2.3 equiv),
in pyridine, ii) 4,4-dimethoxytrityl chloride (2.3 equiv), in pyridine,
iii) 4,4-dimethoxytrityl chloride (2.7 equiv), 4-(dimethyl-
amino)pyridine (0.3 equiv), in pyridine, room temperature, 16 h, 40%.
d) Trisopropylsilyl chloride (4 equiv), AgNO_3 (4 equiv), 18 h, 26%.
e) 2-Cyanoethyl N,N-disopropylchlorophosphoramidite (3 equiv), N,N-
dimethylthylamine (10 equiv), in dichloromethane, room temperature, 105 min,
44%. (B) Reagents and conditions: a) aqueous ammonia, microwave, 140 °C, 2 h, 90%.
NMR spectroscopy which was consistent with the structure of the desired 8-9N isomer 4, indicated by strong ROEs of the nucleobase C3'-H with ribose C2'-H, C2'-H and C1'-H (see Supporting Information File 1). The benzoyl groups of nucleoside 4 were then cleaved with methylene in ethanol and water to furnish the free c3A nucleoside 5. An authentic reference sample that was synthesized according to the previously established 12-step route was used for direct spectroscopic comparison (see Supporting Information File 1) and additionally confirmed its identity. Then, treatment with N,N-dibutylformamide dimethyl acetal [31] resulted in amidine protection of the exocyclic C6-NH2 group. At the same time, the applied excess of the reagent allowed to transiently form the corresponding nucleoside 2',3'-O-acetal [32], leaving the primary 5'-OH group available for selective tritylation with 4,4'-dimethoxytrityl chloride to give compound 6. Selective protection of the 2'-OH was challenging. Initial attempts that focused on the introduction of the TBDMS group according to the procedure described by McLaughlin and co-workers [17] were unsuccessful. Also, attempts to introduce the [trisopropylsilyl]oxymethyl group (TOM) following standard procedures [32] unfortunately failed. We encountered these problems already in our previously published synthesis for N6-benzoyl protected c3A phosphoramidite [16], and therefore, we decided to apply trisopropylsilyl chloride (TIPS-Cl) and silver nitrate which resulted in the desired 2'-O-TIPS protected nucleoside 7 in 28% yield after chromatographic separation from the corresponding 3'-regiosomer. Finally, the 5'-O-DMTr-2'-O-TIPS protected 3-deazaadenosine derivative 7 was converted into the phosphoramidite building block 8 with 2-cyanoethyl diisopropylchlorophosphoramidite in the presence of N-dimethylformamide. Starting from compound 3, our route provides 8 in a 6% overall yield in five steps with four chromatographic purifications; in total, 0.6 g of 8 was obtained in the course of this study.

**Conclusion**

With the reported 5-step synthesis of a c3A phosphoramidite we created a route that is superior to previously described syntheses in terms of efficacy and ease of laboratory handling. The key reaction is a silyl-Hilbert-Johnson nucleosidation using unprotected 6-amino-3-deazapurine and benzoyl-protected 1-O-acetylibrobose, providing 3-deazaadenosine (c3A) in high yields for the subsequent functionalizations to yield a properly protected building block for RNA solid-phase synthesis.

The so-obtained c3A-modified RNAs are currently used for atomic mutagenesis experiments to explore mechanistic aspects of phosphodiester cleavage of recently discovered ribozyme classes, such as twist, pistol, and hatchet ribozymes [1,2,33].

**Experimental**

**General.** Chemical reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich) and used without further purification. 4-Aminomimidazo[4,5-c]pyridine (6-amino-3-deazapurine) and 4-chloromimidazo[4,5-c]pyridine (6-chloro-3-deazapurine) were purchased from Synthionix and Carbogen. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4 Å). The reactions were carried out under an argon atmosphere. Analytical thin-layer chromatography (TLC) was carried out on Marchery-Nagel Polygram SIL G/UV254 plates. Column chromatography was carried out on silica gel 60 (70–230 mesh). 1H, and 13C NMR spectra were recorded on Bruker DRX 300 MHz and Bruker Avance II 600 MHz instruments. Chemical shifts (δ) are reported relative to tetramethylsilane (TMS) and referenced to the residual proton or carbon signal of the deuterated solvent: CDCl3 (7.26 ppm) or DMSO-d6 (2.49 ppm) for 1H NMR; CDCl3 (77.0 ppm) or DMSO-d6 (39.5 ppm) for 13C NMR spectra. 1H and 13C assignments are based on COSY and HSQC experiments. MS experiments were performed on a Waters ESI TOF LCT Premier Serie KD172 or Bruker 7T FT-ICR instrument with an electrospray ion source. Samples were analyzed in the positive-ion mode.

**2',3',5'-Tri-O-benzoyl-3-deazaadenosine (4).** 1-O-Acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (1.1360 g, 2.25 mmol) and compound 3 (303.6 mg, 2.26 mmol) were suspended in dry toluene (50 mL) under an argon atmosphere, after which N,O-bis(trimethylsilyl)acetamide (1.92 mL, 7.85 mmol) was added at room temperature. The suspension was heated up and kept at 105 °C for 2.5 h and then cooled to room temperature, resulting in a clear beige solution. Trimethylsilyl trifluoromethanesulfonate (1.22 mL, 6.72 mmol) was added and the solution was stirred for 1 h at 105 °C, followed by evaporation of all volatiles. The residue was diluted in dichloromethane (20 mL) and washed with saturated sodium bicarbonate solution (3 × 30 mL). The combined organic layers were dried over Na2SO4 and all volatiles were evaporated. The yellow-beige crude product was purified by flash chromatography (1% methanol in dichloromethane + 1.5% triethylamine, size: 18.0 × 2.5 cm, 23 g silica gel). Yield: 823.7 mg (63%) of compound 4 as beige foam. TLC (10% methanol in dichloromethane): RF = 0.52; 1H NMR (300 MHz, DMSO-d6) δ 4.80-4.70 (m, 2H, H-C(5')), 4.84-4.86 (t, J = 3.7 Hz, 1H, H-C(4')), 5.96-5.99 (t, J = 5.3 Hz, 1H, H-C(3')) 6.03-6.07 (t, J = 6.1 Hz, 1H, H-C(2')) 6.25 (s, 2H, NH2) 6.57-6.59 (d, J = 5.9 Hz, 1H, H-C(1')) 6.93-6.95 (d, J = 5.8 Hz, 1H, H-C(3)), 7.39–7.72 (m, 10H, H(benzoyl), 7.83–7.86 (d, J = 7.5 Hz, 2H, H(benzoyl)), 7.97–8.04 (q, J1 = 13.8 Hz, J2 = 7.6 Hz, 4H, H(benzoyl)), 8.38 (s, 1H, H-C(8)) ppm; 13C NMR (75 MHz, DMSO-d6) δ 63.67 (C-(5')), 70.54 (C-(3')), 72.83 (C-(2')), 79.37 (C-(4')) 86.53 (C-(1')) 96.98 (C-(3)), 125.19, 149.82 ppm (C-(2)), 159.16 ppm (C-(5)).
126.76, 128.08, 128.52, 128.65, 128.69, 128.77 (C(benzoyl)), 129.16, 129.22, 129.29, 129.39 (C(benzoyl)), 133.57, 133.86, 133.95 (C(benzoyl)), 137.12, 139.98 (C-(8)), 140.98 (C-(2)), 152.55, 164.31, 164.70, 165.45 ppm; HRMS [M + H$^+$]: calcd for C$_{32}$H$_{58}$N$_4$O$_7$, 579.1880; found, 579.1852.

3-Deazaadenosine (5). Compound 4 (1.231 g, 2.13 mmol) was dissolved in a solution of 33% methanamine in ethanol (10 mL) and 40% methanamine in water (10 mL) and stirred for 18 hours at room temperature. All volatiles were evaporated and the residue was dried in high vacuum. The crude product was purified by dissolving the byproduct (N-methylbenzamide) in chloroform and subsequent collection of the precipitate by centrifugation (3000 rpm, rt, 1 min). Yield: 537.1 mg (95%) of compound 5 as beige solid. TLC (30% methan in dichloromethane): $R_1 = 0.23$; $^{1}H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 3.57–3.68 (q, $J = 10.1$ Hz, 2H, H-(C(5))), 3.94–3.96 (d, $J = 3.3$ Hz, 1H, H-(C(4'))), 4.08–4.10 (d, $J = 3.1$ Hz, H-C(3')), 4.30–4.32 (d, $J = 5.5$ Hz, 1H, H-C(2')), 5.06–5.09 (t, $J = 6.5$ Hz, 1H, OH-C(1')), 5.19–5.20 (d, $J = 4.0$ Hz, 1H, OH-C(3')), 5.44–5.46 (d, $J = 6.2$ Hz, 1H, OH-C(2')), 5.45–5.46 (d, $J = 6.2$ Hz, 1H, H-C(1')), 6.17 (br, 2H, NH$_2$), 6.90–6.92 (d, $J = 5.8$ Hz, 1H, H-C(3)), 7.65–7.67 (d, $J = 5.8$ Hz, 1H, H-C(2)), 8.29 (s, 1H, H-C(8)) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 46.21 (C-(5')), 47.08 (C-(3')), 73.86 (C-(2')), 85.53 (C-(4')), 88.66 (C-(1')), 97.37 (C-(3')), 126.91, 137.58, 139.95 (C-(8)), 140.45 (C-(2')), 152.36 ppm; HRMS [M + H$^+$]: calcd for C$_{11}$H$_{14}$N$_4$O$_4$$^+$, 267.1093; found, 267.1074.

N$^6$-[{Dibutylamino)methylene]-5'-O-(4,4'-dimethoxytrityl)-3-deazaadenosine (6). Compound 5 (199 mg, 747 mmol) was weighed into a 25 mL two-necked flask and dried in high vacuum for 1 hour. Under an argon atmosphere dry pyridine (5 mL) and N,N-dibutylformamide dimethyl acetal (0.40 mL, 1.74 mmol) were added and stirred for 1.5 h at room temperature. Afterwards, all volatiles were evaporated, the residue dried in high vacuum and redissolved in dry pyridine (5 mL). 4-(Dimethylamino)pyridine (28.4 mg, 233 mmol) and 4,4'-dimethoxytrityl chloride (686 mg, 2.03 mmol) were added and the reaction was allowed to proceed for 16 hours. The reaction was quenched with methanol (1 mL) and all volatiles were evaporated, followed by coevaporation with toluene (2 × 10 mL). The residue was partitioned between dichloromethane (10 mL) and 5% aqueous citric acid solution (7 mL). The organic layer was separated, washed with water and saturated sodium bicarbonate solution (10 mL each), dried over Na$_2$SO$_4$ and evaporated. The crude product was purified by flash chromatography (1% methanol in dichloromethane + 1.5% triethylamine, size: 18.0 × 2.0 cm, 21 g silica gel). Yield: 212 mg (40%) of compound 6 as white foam. TLC (10% methanol in dichloromethane): $R_1 = 0.32$; $^{1}H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.89–0.96 (q, 6H, NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 1.28–1.37 (sexet, $J = 6.8$ Hz, 4H, NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 1.53–1.64 (quinet, $J = 7.8$ Hz, 4H, NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 3.22–3.27 (m, 2H, H-C(5')), 3.33–3.40 (t, $J = 7.3$ Hz, 4H, NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 3.72 (s, 6H, 2 × OCH$_3$), 4.09–4.10 (d, $J = 4.1$ Hz, 1H, H-C(4')), 4.19–4.21 (m, 1H, H-C(3')), 4.45–4.46 (m, 1H, H-(C-2')), 5.25–5.27 (m, 1H, OH-C(3'))), 5.60–5.65 (m, 1H, OH-C(2')), 5.86–5.87 (d, $J = 5.2$ Hz, 1H, H-C(1')), 6.81–6.84 (d, $J = 6.8$ Hz, 4H, H(ar)-DMMTr), 7.20–7.29 (m, 8H, H(ar)-DMMTr, H-C(2')), 7.34–7.36 (d, $J = 7.4$ Hz, 2H, H(ar)-DMMTr), 7.78–7.80 (d, $J = 5.6$ Hz, 1H, H-C(3)), 8.24 (s, 1H, NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 8.64 (s, 1H, H-C(8)) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 13.61, 13.82 (NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 19.21, 19.73 (NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 28.78, 30.74 (NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 44.06 (C-(5')), 50.46, 52.07 (NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 55.01 (2 × OCH$_3$ (DMMTr)), 63.57, 70.11 (C-(3')), 73.40 (C-(2')), 83.38 (C-(4')), 85.66, 89.00 (C-(1')), 101.66 (C-(3)), 112.88, 113.19 (C(ar)), 126.68, 127.71, 127.83, 129.71, 133.24 (C-(ar)), 135.34, 135.39, 138.92, 140.22 (C-(2)), 140.83 (NCH$_2$(CH$_2$CH$_2$CH$_2$CH$_2$)), 144.73, 155.05, 155.88, 158.08 (C-(8)) ppm; HRMS [M + H$^+$]: calcd for C$_{44}$H$_{40}$N$_4$O$_6$$^+$, 708.3761; found, 708.3766.
(d, J = 7.7 Hz, 4H, H(ar)-DMTr), 7.17–7.28 (m, 8H, H(ar)-DMTr, H-C(2)), 7.34–7.37 (d, J = 8.0 Hz, 2H, H(ar)-DMTr), 7.63–7.65 (d, J = 5.3 Hz, 1H, H-C(3)), 8.26 (s, 1H, NCH(N(CH₂CH₂CH₃)₃)₂), 8.59 (s, 1H, H-C(8)) ppm; ¹³C NMR (150 MHz, CDCl₃) δ 12.01, 13.77, 14.02 (Si(CH₃)₃), 17.49, 17.67 (NCH(N(CH₂CH₂CH₃)₂), 19.88, 20.27 (NCH(N(CH₂CH₂CH₃)₂), 29.30, 31.22 (NCH(N(CH₂CH₂CH₃)₂), 44.82 (NCH(N(CH₂CH₂CH₃)₂), 51.37 (Si(CH₃)₃), 55.23, 63.51 (C₅(−)), 71.75 (C₃′), 75.57 (C₂′), 83.97 (C₄′), 86.99, 89.17 (C₁′), 101.57 (C₂), 113.26, 127.05, 127.97, 128.16, 130.09, 130.16 (C₉-ar-DMTr), 133.63, 135.29, 135.38, 139.03, 140.49 (NCH(N(CH₂CH₂CH₃)₂), 141.42 (C₃′), 144.37, 155.87, 156.29 (C₅(−)), 158.63 ppm; HRMS [M + H⁺]: calculated for C₁₀H₁₀N₂O₅PSi₁²⁺: 1064.6168; found, 1064.6192.

Supporting Information
Supporting Information File 1
Synthetic procedures of compounds 1–3 and NMR spectra of compounds 1–8.
[http://www.beilstein-journals.org/bjoc/content supplementary/1860-5397-12-250-S1.pdf](http://www.beilstein-journals.org/bjoc/content supplementary/1860-5397-12-250-S1.pdf)

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