Modified GSMP Synthesis Greatly Improves the Disulfide Crosslink of T7 Run-Off siRNAs with Cell Penetrating Peptides

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Abstract: Preventing intramolecular cyclization greatly improves 5′-deoxy-5′-thioguanosine monophosphorothioate (GSMP) synthesis and its application as a potent initiator nucleotide for T7 run-off transcription of noncoding RNAs. GSMP was efficiently incorporated into the 5′-end of siRNA sense strands and the resulting thiol-modified siRNA was crosslinked with a free cysteine of cell penetrating peptide Penetratin as monitored by mass spectrometry. Cellular uptake and the knockdown potential of the peptide-coupled siRNA (pepsiRNA) were evaluated in primary cells.

Key words: guanosine, T7 RNA, siRNA, Penetratin, disulfide crosslink, knockdown

Over the past decade, RNA-based techniques such as antisense-mediated silencing,[1] RNA-aptamers,[2] and RNA interference (RNAi)[3] have become important tools for regulation of gene function. Thus, there is a great interest in noncoding RNA functionalization in order to immobilize RNA to solid phase for *in vitro* evolution or to covalently couple reporter molecules, ligands for targeting or delivery vectors as functional molecules.[4] Especially, the transfection of certain mammalian cell lines and primary cells with RNAs can be cumbersome. Previously, cell-penetrating peptides (CPPs) were coupled to siRNAs to enhance cellular uptake.[5]

For this kind of delivery strategy the functionalization of the RNA with thiol groups is particularly attractive since they react with the thiol group of cysteine-modified CPPs under the formation of disulfide bonds.[6] Once these siRNA-conjugates reach the interior of a cell, the disulfide bonds are cleaved under the reducing conditions of the cytosol and siRNA is released from the delivery peptide. In our approach, we chose an enzymatic route to thiol-modified siRNAs for the coupling to CPPs. This strategy can be adapted to the peptide coupling of other noncoding RNAs. If RNA is transcribed from a DNA template with T7 RNA polymerase, the 5′-terminus of the RNA can be modified by the use of guanosine monophosphate (GMP) derivatives that act as initiator nucleotides and are selectively incorporated at the 5′-terminus.[7] GMP derivatives are favored as guanosine is the best enhancer of the initiation rate of T7 in vitro transcription. RNA modified with guanosine monophosphorothioate (GMPS) can be directly coupled with thiopyridyl-activated compounds[8] to form less stable phosphorothioate sulfides (R-SSPO$_3$-RNA).[9] Conversely, modification with 5′-deoxy-5′-thioguanosine monophosphorothioate (GSMP)[4a] leads to a 5′-terminal phosphate, which needs to be removed by a phosphatase to liberate the thiol function. The resulting 5′-HS-RNA forms more stable disulfide bonds upon coupling. At the same time this approach provides a minimal distance between the cargo molecule and the RNA (R-S-S-RNA).

The preparation of GSMP has been described as a four-step synthesis (Scheme 1).[4a] However, the fast intramolecular cyclization of the iodinated isopropylideneguanosine **3** leads to a severe decrease in yields of the unprotected 5′-desoxyguanosine. Another route to 5′-deoxy-5′-iodoguanosine, which is stable against this side reaction, is based on the iodination of the 5′-position without the protection of the 3′- and 2′-OH groups.[10] Although this synthesis has been described in several examples with yields in the range of 70%, it suffers from the difficulty to isolate a pure product. In our approach we introduced an additional protection step to reduce the rate of the side reaction and used the GSMP *in vitro* transcription strategy to generate 5′-SH-functionalized siRNAs. Finally, the overall yield of 5′-desoxy-5′-iodoguanosine is improved compared to the other approaches. Furthermore, the formation of susceptible intermediates or purification problems is avoided. Following the previously reported synthesis[4a] guanosine (**1**) was protected at its 2′- and 3′-hydroxy groups by an acetal to produce 2′,3′-*O*,*O*-isopropylideneguanosine (**2**) in 94% yield. Reaction of the 5′-hydroxy group of **2** with methyltriphenoxyphosphonium iodide (MTPI) in a Moffat reaction[11] afforded the crude 5′-deoxy-5′-iodo-2′,3′-*O*,*O*-isopropylideneguanosine (**3**) as a sticky paste leading to a yield of only 39%. However, 2′,3′-*O*,*O*-isopropylidene-*N*-3,5′-*C*-cycloguanosine (**6**) was formed as a by-product to **3**. This type of cyclization has been described previously for related compounds (Scheme 1).[11,12] To prevent the cyclization and to stabilize the product, we protected the primary amino group by for-

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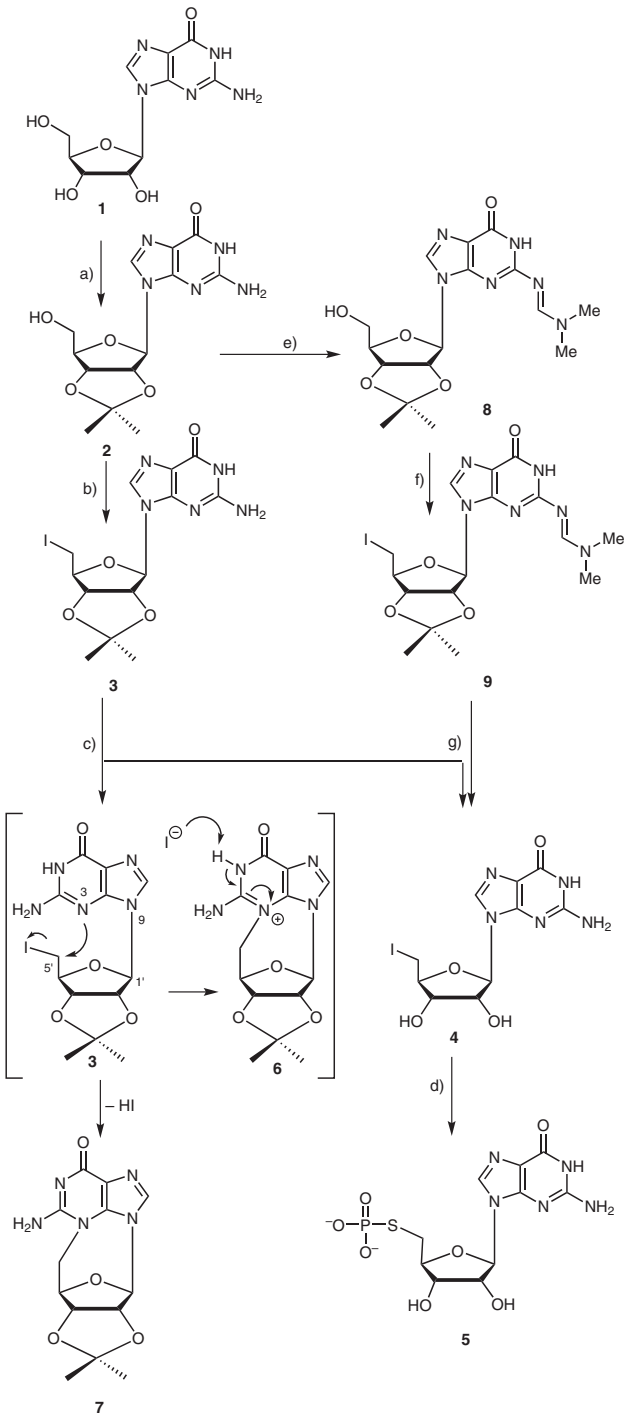
mation of an imine with dimethylformamide dimethyl acetal,[13] which reduced the flexibility of the purine and thereby the rate of intramolecular nucleophilic attack. 2-*N*,*N*-Dimethylaminomethylene-2′,3′-*O*,*O*-isopropylideneguanosine (**8**) was obtained in 80% yield and could be iodinated to yield 2-*N*,*N*-dimethylaminomethylene-2′,3′-*O*,*O*-isopropylidene-5′-deoxy-5′-iodoguanosine (**9**) in 99% yield.[14] The dried compound **9** could be stored for four weeks at 4 °C without intramolecular cyclization.



Scheme 1 Modification of GSMP synthesis.[7] *Reagents and conditions*: (a) acetone, cat. $HClO_4$, 4 h, r.t.; (b) MTPI, THF, 30 min, –65 °C, 4 h, r.t.; (c) 50% HCOOH, 3 d; (d) Na_3PO_3S, H_2O, 3 d, r.t.; cyclization of **3** generates 2′,3′-*O*,*O*-isopropylidene-3,5′-C-cycloguanosine (**7**) via a charged intermediate (**6**).[11] Modified synthesis of GSMP. *Reagents and conditions*: (e) $(MeO)_2HCNMe_2$, DMF, 4 h, 50 °C; (f) MTPI, THF, 30 min, –65 °C, 4 h, r.t.; (g) 50% HCOOH.

The rate of cyclization for the unprotected **3** and protected iodide **9** was monitored by ^{1}H NMR in DMSO-d_6 at ambient temperature (Figure S1, Supporting Information). For **3**, ^{1}H NMR spectra were recorded every 30 minutes revealing a first order kinetic with $k = 0.181 \pm 0.008$ h^{-1} while recording ^{1}H NMR spectra for protected iodide **9** every 24 hours yielded a first-order rate constant of $k = 0.0145 \pm 0.0008$ h^{-1} corresponding to an approximately 12-fold decrease in reaction rate (Figure 1).

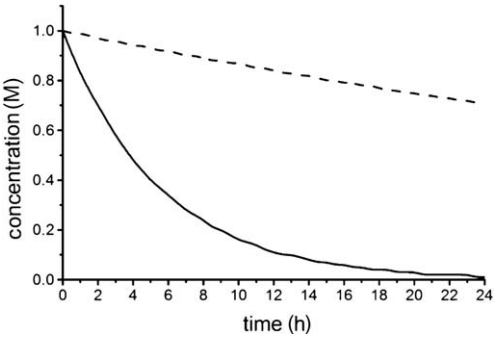


Figure 1 Introduction of an *N*,*N*-dimethylaminomethylene protecting group increased the half life time of **3** by a factor of 12. Averages of significant integrals for **9** (dashed) and unprotected **3** (solid) are plotted against time. Both sets of data are fitted with a first-order kinetic with $k = 0.0145$ h^{-1} and $k = 0.181$ h^{-1}, respectively.

Both protecting groups, the acetal and the imine, could be removed from **9** by stirring in 50% formic acid for three days at room temperature yielding 5′-deoxy-5′-iodoguanosine (**4**) without formation of the cyclization product **7**. Compound **4** was treated with a 2–3-fold molar excess of sodium thiophosphate in degassed water under argon to yield 5′-deoxy-5′-thioguanosine monophosphorothioate

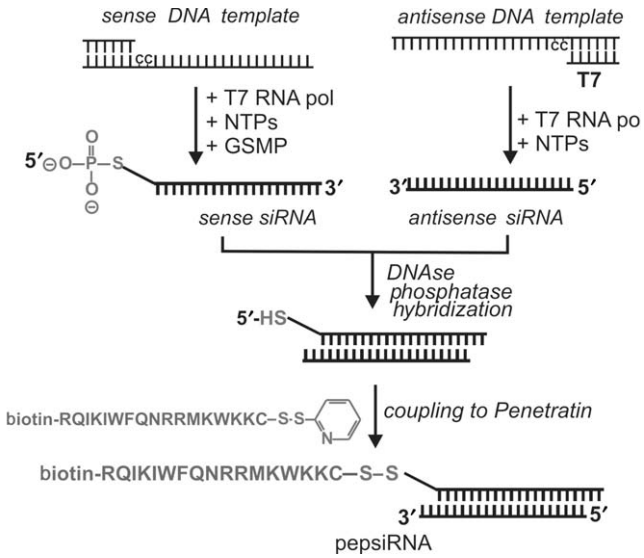


Figure 2 Schematic view of the enzymatic synthesis of thiol-modified siRNAs and their coupling to the cell penetrating peptide Penetratin

(GSMP; **5**). Yields could be greatly improved by dissolving the thiophosphate in degassed water and removing the precipitate prior to the addition to a suspension of **4**. ^{1}H NMR and ^{31}P NMR spectroscopy indicated quantitative formation of GSMP (**5**) (δ = 19 ppm) (Figure S1, Supporting Information). After precipitation of the remaining thiophosphate the crude product was purified by gravity chromatography on RP-18 with water to afford pure GSMP in 68% yield. To prove its suitability as an initiator nucleotide, purified GSMP was then used for the enzymatic synthesis of 5′-thiolated siRNAs (Figure 2). SiRNAs were generated by T7-based *in vitro* transcription of DNA oligonucleotides encoding the appropriate 21-nt sequences of the respective target genes. The DNA templates comprised a T7 recognition sequence at the 5′-end, which was hybridized with a matching oligonucleotide to provide a partially double-stranded recognition site for T7-polymerase. *In vitro* transcription led to RNA-transcripts complementary to the single stranded DNA-sequence containing a guanosine at the 5′-end and a 2-nt 3′-TT overhang. For the generation of 5′-thiol-modified siRNAs an eight-fold excess of GSMP (**5**) was added to the sense strand reaction mixture. Following transcription, hybridization, and treatment with DNase to degrade residual DNA template and calf intestinal phosphatase to remove the 5′-phosphate of the GSMP, the disulfide-linked dimers were reduced with DTT. Eventually, the 5′-SH-siRNAs were incubated with dithiodipyridyl-activated Penetratin to form conjugates in a thiol-exchange reaction to selectively form heterodimers.[15]

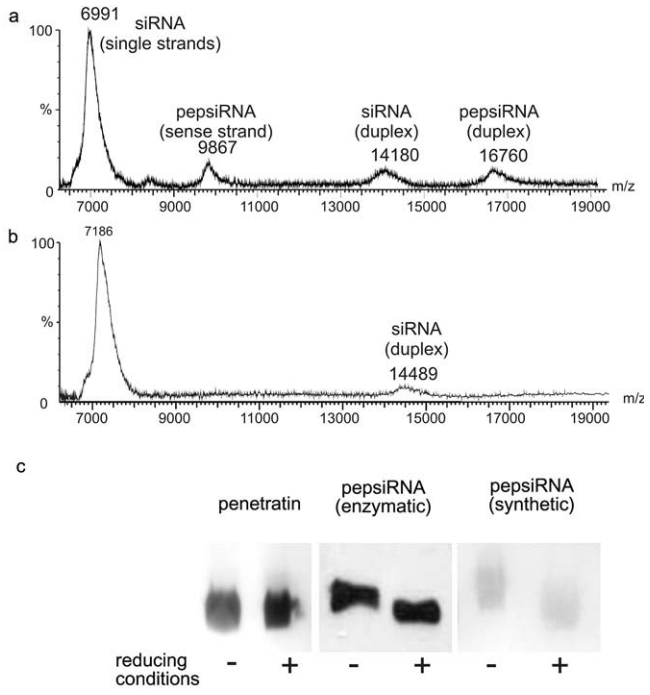


Figure 3 MALDI and SDS-PAGE analysis of siRNA-Penetratin conjugate. (a) MALDI spectrum of pepsiRNA (GFP) duplex before and (b) after reduction with DTT. The spectra (negative mode, matrix: THAP, NH_4Ac added) show four peaks corresponding to ss-siRNA (ca. 7kD), pepsiRNA (sense strand) (ca. 9.8 kD), siRNA duplex (ca. 14 kD) and pepsiRNA duplex (ca. 16.8 kD, ca. 8.4 kD). (c) 15%-SDS-PAGE analysis of Penetratin (left panel), pepsiRNAs derived from a synthetic 5′-SH-C_6-siRNA (right panel) and the corresponding enzymatically synthesized 5′-SH-siRNA (middle panel) under non-reducing and reducing conditions.

The ratio of modified to unmodified RNA was determined by a gel shift assay (see Supporting Information). The resulting peptide-coupled siRNAs (pepsiRNAs) were characterized by MALDI mass spectrometry and SDS-PAGE under reducing and non-reducing conditions (Figure 3). Finally, the enzymatically prepared pepsiRNAs were tested for their ability to mediate RNAi in mammalian cell culture in comparison to conjugates of synthetic thiolated siRNA with Penetratin. Murine primary fibroblasts and myogenic cells were obtained from a transgenic GFP mouse,[16] and down-regulation of GFP upon treatment with 10–25 nM pepsiRNAs was measured by live microscopy after 48 hours (Figure 4). Both synthetic 5′-SH-C_6-modified siRNA and the enzymatically generated siRNA induced a knockdown of GFP to about 35%.

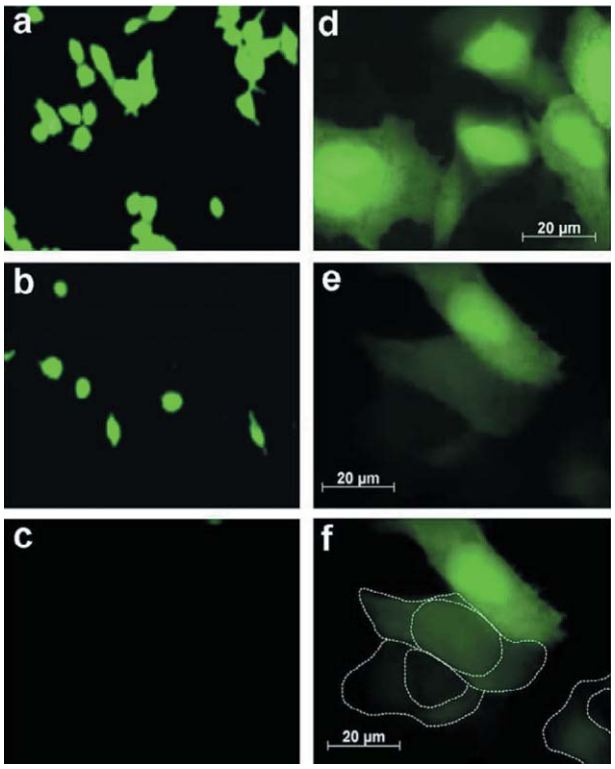


Figure 4 RNA interference by synthetic and enzymatic pepsiRNAs. Primary myogenic cells from skeletal muscle (a–c) and primary murine fibroblasts (d–f) of transgenic GFP mice were treated with 10 nM (b) and 25 nM (c) of pepsiRNA (synthetic) against GFP and 10 nM (e, f) of pepsiRNA (enzymatic). Fluorescent micrographs were taken 72 h post treatment using the appropriate GFP/FITC filter. (f) The dashed line shows the surrounding of the cells that exhibit a knockdown of GFP.

We also synthesized pepsiRNAs against glucosylceramide synthase (GCS), an endogenous gene, which is involved in the synthesis of glucosylceramide (GlcCer). GlcCer is essential for the transport of tyrosinase to the melanosomes, where it converts tyrosine into L-DOPA in the first rate-limiting step of melanine synthesis and pig-

mentation.[17] Down-regulation of pigmentation was monitored as an indicator of GCS silencing 2–4 days after treatment of murine melanoma cells. Knockdown of GCS to below 10% was determined by mRNA quantification and by Western blot analysis, and could be observed as a loss of pigmentation in melanoma cells (Figure 5). These model experiments show that our alternative strategy leads to functional peptide-coupled siRNAs that are suitable for the treatment of primary mammalian cells.

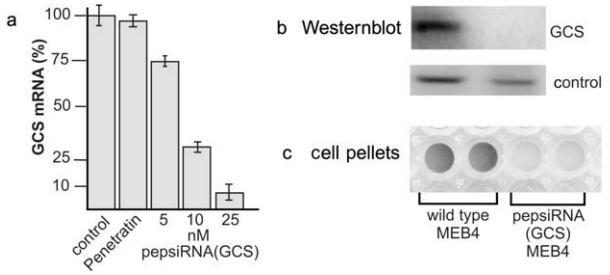


Figure 5 RNAi with pepsiRNA(GCS) in murine melanoma cells. (a) pepsiRNA (5–25 nM) against GCS was added to MEB4 cells. As controls, cells were treated with 100 nM uncoupled Penetratin. The data were averaged from five independent experiments +/– s.d. GCS mRNA levels were measured by RT-PCR (a) as well as by Western blot (for 25 nM pepsiRNA) (b). Ratios were normalized to the non-treated cells. (c) Visual assay: MEB4 cells (1×10^7) were treated with the pepsiRNA (25 nM) against GCS. The color of the harvested cells shows a lack of pigmentation in GCS-deficient cells.

In conclusion, we have demonstrated an alternative route to peptide-coupled noncoding RNAs using GSMP as an initiator nucleotide in T7 *in vitro* transcription. As an example we generated siRNAs with a thiol modification for disulfide crosslinking with a cell penetrating peptide. The so-called pepsiRNAs have been shown to be suitable for their use in RNAi experiments. The strategy was developed due to shortcomings in the synthesis of GSMP. Formation of cyclization by-products and lack of solubility of the precursor reduce the overall yield. The synthesis was greatly improved by the introduction of an imine protecting group on the purine base preventing intramolecular cyclization. With an optimized purification protocol for GSMP we achieved an overall yield of 53% in five steps as opposed to the reported 35% in four steps.[4a]

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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(14) ***N*,*N*-Dimethylaminomethylene-2′,3′-*O*,*O*-isopropylideneguanosine (8)**: 2′,3′-*O*,*O*-Isopropylideneguanosine (**2**; 5.75 g, 17.8 mmol) was resuspended in DMF (60 mL) and *N*,*N*-dimethylformamide dimethyl acetal (8.91 mL) was added under argon to yield an orange-brown solution. The reaction mixture was stirred at 50 °C for 4 h. The solvent was removed under reduced pressure and at elevated temperatures (ca. 55 °C), the white residue was then removed by filtration. The filtrate was dried under reduced pressure, redissolved in MeOH (25 mL) yielding a fluorescent green solution and precipitated with 50 mL of EtOAc. After storage overnight at 4 °C, the residue was removed by filtration and the combined filtrates were thoroughly washed with EtOAc and dried under reduced pressure. The product was obtained as a white powder in 80% yield (5.38 g, 14.2 mmol). All steps were carried out under protection from light; mp >200 °C; R_f 0.47 ($CHCl_3$–MeOH, 8:1), 0.08 ($CHCl_3$–MeOH, 19:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.32 (br s, 1 H, NH), 8.56 (s, 1 H, H-imine), 8.00 (s, 1 H, H-8), 6.03 (d, *J* = 3.0 Hz, 1 H, H-1′), 5.26 (dd, *J* = 3.0, 6.3 Hz, 1 H, H-2′), 5.03 (dd, *J* = 5.4, 5.4 Hz, 1 H, 5′-OH), 4.95 (dd, *J* = 2.9, 6.3 Hz, 1 H, H-3′), 4.13 (ddd, *J* = 2.9, 4.9, 4.9 Hz, 1 H, H-4′), 3.59–3.47 (m, 2 H, H-5′), 3.15 (s, 3 H, NMe), 3.03 (s, 3 H, NMe), 1.53 (s, 3 H, Me), 1.33 (s, 3 H, Me). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 158.3 (C-imine), 157.6, 157.5 (C-4, C-6), 149.6 (C2), 136.0 (C-8), 119.9 (C-5), 113.2 (C-quat), 88.6, 86.4, 83.6, 81.2 (C-1′ to C-4′), 61.5 (C-5′), 40.8 (NMe), 34.8 (NMe), 27.2 (Me), 25.3 (Me). MS (EI; 70 eV): *m*/*z* (%) = 378 (89%) [M]$^+$, 363 (6%) [M – CH]$^+$, 348 (6%) [M – 2 Me]$^+$, 333 (2%) [M – NH(Me)$_2$]$^+$, 206 (100%) [M – DAMG]$^+$, 191 (26%) [DAMG – Me]$^+$, 176 (3%) [DAMG – 2 Me]$^+$, 150 (6%) [guanine]$^+$. HRMS: *m*/*z* calcd for $C_{16}H_{22}N_6O_5$: 378.1652; found: 378.1654 (±0.0095); (DAMG = 2-*N*′,*N*-dimethylaminomethylene guanine).
***N*,*N*-Dimethylaminomethylene-2′,3′-*O*,*O*-isopropylidene-5′-deoxy-5′-iodoguanosine (9)**: Ground **8** (2.20 g,

5.81 mmol) was resuspended in anhyd THF (110 mL) under argon and cooled to –70 °C by a mixture of acetone and dry ice. Methyltriphenoxyphosphonium iodide (3.94 g, 8.71 mmol; 1.5 equiv) was added. Due to the light sensitivity of the reactant and the product all subsequent steps were carried out under exclusion of light. After 30 min of stirring the reaction mixture was allowed to warm to r.t. and stirred for another 4 h. The reaction was stopped by the addition of MeOH (5 mL) and the solvent was removed under reduced pressure. The dark red residue was dissolved in MeOH–$CHCl_3$ (1:4; 2.5 mL) and $CHCl_3$ (7 mL) was added. The solution of the crude product was subjected to chromatography on silica gel (Merck 0.40–0.65 μm; eluent: $CHCl_3$–MeOH, 9:1). After removal of the solvent, **9** was obtained as an orange solid in 99% yield (2.81 g, 5.75 mmol); mp 95–97 °C; R_f 0.74 ($CHCl_3$–MeOH, 5:1), 0.22 ($CHCl_3$–MeOH, 19:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.39 (br s, 1 H, NH), 8.58 (s, 1 H, H-imine), 8.01 (s, 1 H, H-8), 6.15 (d, *J* = 2.1 Hz, 1 H, H-1′), 5.42 (dd, *J* = 2.1, 6.3 Hz, 1 H, H-2′), 5.02 (dd, *J* = 2.8, 6.3 Hz, 1 H, H-3′), 4.28 (ddd, *J* = 2.8, 5.7, 9.0 Hz, 1 H, H-4′), 3.49 (dd, *J* = 9.0, 9.9 Hz, 1 H, H-5′), 3.30 (dd, *J* = 5.7, 9.9 Hz, 1 H, H-5′), 3.19 (s, 3 H, NMe), 3.04 (s, 3 H, NMe), 1.53 (s, 3 H, Me), 1.35 (s, 3 H, Me). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 158.2 (C-imine), 157.7, 157.5 (C-4, C-6), 149.2 (C2), 137.9 (C-8), 120.2 (C-5), 113.5 (C-quart), 89.4, 86.4, 84.0, 83.9 (C-1′ to C-4′), 41.1 (NMe), 34.9 (NMe), 27.2 (Me), 25.3 (Me), 6.7 (C-5′). MS (EI; 70 eV): *m*/*z* = 488 (13%) $[M]^+$, 361 (7%) $[M – I]^+$, 360 (35%) $[M – HI]^+$, 345 (1%) $[M – HI, Me]^+$, 206 (3%) $[DAMG]^+$, 188 (2%) $[DAMG – H_2O]^+$, 176 (26%) $[DAMG – 2 Me]^+$, 149 (12%) $[guanine – H]^+$, 128 (100%) [HI]. HRMS: *m*/*z* calcd for $C_{16}H_{21}N_6O_4I$: 488.0669; found: 488.06631; (DAMG = 2-*N*′,*N*-dimethylaminomethylene guanine).

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