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Sulfonamide bearing oligonucleotides: Simple synthesis and efficient RNA recognition

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ABSTRACT

Four pyrimidine nucleosides wherein a benzensulfonamide group is linked to the C-5 position of the uracil nucleobase through a triazolyl or an alkynyl linker were prepared by Cu(I)-assisted azide-alkyne cycloadditions (CuAAC) or Sonogashira reactions, respectively, and incorporated into oligonucleotides. Efficient π–π-stacking between two or more phenyltriazoles in the major groove was found to increase the thermal stability of a DNA:RNA duplex significantly. On the other hand, the alkynyl group was not as efficient in stacking as the triazolyl group. No effect of positional orientation of the sulfonamide group on the stacking efficiency was observed, and the most stable DNA:RNA duplex contained four consecutive sulfonamide substituted phenyltriazole moieties in the major groove.

1. Introduction

The era of nucleic acid therapies was born in 1978 with the proof of concept for the antisense mechanism whereby a string of DNA nucleotides could be used to bind and block mRNA function.[1] Thus, antisense technology presents an opportunity to disrupt the gene expression at the translational level by an accurate knowledge of the target mRNA sequence and rational design of its complementary antisense oligonucleotide sequence having higher affinity for RNA so as to form a stable DNA:RNA duplex.[2] The idea of taking advantage of efficient π–π-stacking for increasing the duplex stability has been exploited by us[3–5] and others.[6–8] We reported that a triazole group attached to the 5-position of dU projects itself in the major groove of DNA:DNA or DNA:RNA duplexes and can lead to increased thermal stabilities through efficient π–π-stacking.[3] Furthermore, only two consecutive incorporations were found to be sufficient to give significant DNA:RNA duplex stabilization due to stacking of triazoles and the aromatic substituents attached.[4] The most pronounced effect was given by the monomer containing a phenyltriazole with a distal sulfonamide moiety.[4] Hence a 9-mer DNA:RNA duplex with two *p*-sulfonamidophenyltriazoles (**W** in Scheme 1) in the center displayed an increase in melting temperature of 8 °C as compared to the unmodified duplex while the same 9-mer DNA:DNA duplex exhibited a decrease in thermal melting by 2.5 °C. Molecular modelling studies indicated that the duplex with the sulfonamide monomer demonstrated a perfect stacking of the aromatic ring and a consistent organisation between the neighbouring sulfonamide groups.[4] In another study, we found that phenyltriazoles positioned on the 5-positions of cytosines also can participate in stacking.[5] Importantly, the phenyltriazole modified oligonucleotides were found to be very resistant towards nucleolytic degradation.[5] In the present study, we explore the scope of the stacking effect concerning the positional orientation of a sulfonamide group as well as comparing the stacking potential of the triazole versus an alkynyl linker.

2. Results and discussion

2.1. Chemical synthesis

The C5-triazole-functionalized pyrimidine nucleosides were prepared by following the concept of Click chemistry[9] through Cu(I)-catalyzed azide–alkyne cycloadditions[10] performed on 5′-*O*-DMTr-protected 5-ethynyl-2′-deoxyuridine **1** (Scheme 1) using azides that were prepared and isolated from suitable building blocks (Scheme 2). In this way, nucleoside **2** was made from the protected *p*-azidobenzenesulfonamide **7a** as recently published.[4] The novel *m*-azidobenzenesulfonamide **6b** was prepared by a diazotation of known metanilamide[11] and was protected with a dimethylamidine group to give **7b** following our successful experience

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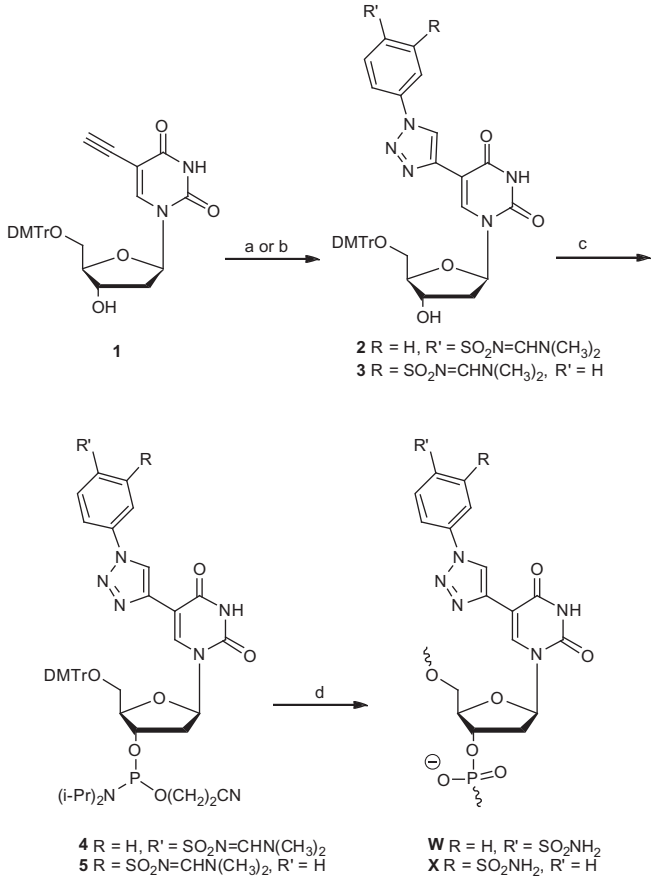
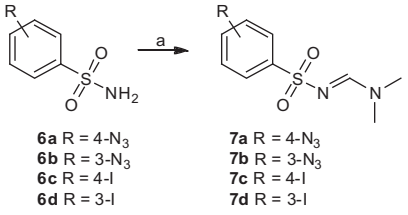


Scheme 1. Reagents and conditions: (a) Ref.4; (b) Azide **7b**, $CuSO_4$, Na ascorbate, *t*-BuOH, H_2O, THF, 51% **3**; (c) $NC(CH_2)_2OPCl(N(iPr)_2)$, $EtN(iPr)_2$, CH_2Cl_2, 67% **4**,[4] 80% **5**; (d) automated DNA-synthesis. DMTr = 4,4′-dimethoxytrityl.

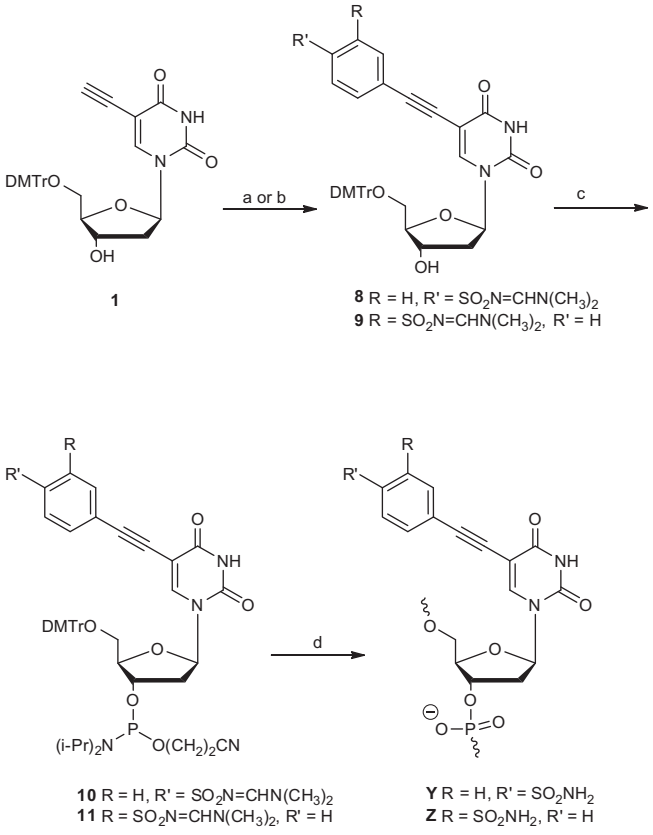


Scheme 2. Reagents and conditions: (a) DMF, $POCl_3$, 73% **7a**,[4] 69% **7b**, 65% **7c**, 82% **7d**.

of using this base-sensitive group for *p*-azidobenzenesulfonamide **6a**.[4] The protected triazole nucleoside **3** was prepared in good yield by the Cu(I)-catalyzed cycloaddition of the protected azide **7b** with **1** (Scheme 1). Both nucleosides **2** and **3** were converted to the corresponding phosphoramidites **4**[4] and **5** in reasonable yields by using a standard method.

The C5-alkynyl-functionalized building blocks **8** and **9** were prepared in good yields through Sonogashira couplings of **1** (Scheme 3) with appropriate protected iodobenzenesulfonamides **7c** and **7d**, which were obtained from the known compounds **6c**[12] and **6d**[13] (Scheme 2). The nucleosides were converted to the corresponding phosphoramidites **10** and **11** in reasonable yields. The identity of the compounds was fully ascertained by NMR (^1H, ^{13}C, ^{31}P, COSY and/or HSQC) and HRMS.

The phosphoramidites **4**,[4] **5**, **10** and **11** were succesfully incorporated into oligodeoxynucleotides using an automated DNA



Scheme 3. Reagents and conditions: (a) Iodophenyl **7c**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, 65% **8**; (b) Iodophenyl **7d**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, 65% **3**; (c) $NC(CH_2)_2OPCl-N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, 67% **10**, 73% **11**; (d) automated DNA-synthesis. DMTr = 4,4′-dimethoxytrityl.

Table 1
Hybridisation data for DNA:DNA duplexes[a]

	T_m (ΔT_m/mod.)[b] (°C)				
	B=	**W**	**X**	**Y**	**Z**
ON1	5′-dGTG TBT TGC	28.0[c] (−5.0)	28.0 (−5.0)	31.5 (−1.5)	31.5 (−1.5)
ON2	5′-dGTG TBB TGC	30.5[c] (−1.3)	29.5 (−1.8)	30.0 (−1.5)	30.5 (−1.3)
ON3	5′-dGTG BBB TGC	33.0[c] (0.0)	32.5 (−0.2)	31.0 (−0.7)	29.5 (−1.2)
ON4	5′-dGTG BBB BGC	35.5[c] (+0.7)	36.5 (+0.9)	31.0 (−0.5)	29.5 (−0.9)

[a] Target sequence 5′-dGCA AAA CAC.
[b] Melting temperatures (T_m values/°C) obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) recorded in a medium salt buffer (Na_2HPO_4 (5 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.5 μM concentrations of each strand. In brackets the changes in melting temperature for each modification **B** (ΔT_m/mod./°C) as compared to the unmodified reference duplex (T_m = 33.0 °C).
[c] Data taken from Ref. 4

synthesizer with tetrazole as the activator. Standard conditions were employed except for extended coupling (15 min) for the modified phosphoramidites. After completion of the synthesis, the oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia. This treatment also removed the amidine protections of the sulfonamides giving the incorporated monomers **W, X, Y** and **Z**, respectively (Schemes 1 and 3). The four monomers were incorporated into the same series of 9-mer oligonucleotides **ON1**–**4** (Tables 1 and 2). The composition and purity (>80%) was verified by MALDI-TOF MS analysis and RP-HPLC, respectively.

Table 2
Hybridisation data for DNA:RNA duplexes[a]

	T_m (ΔT_m/mod.)[b] (°C)				
	B =	**W**	**X**	**Y**	**Z**
ON1	5′-dGTG TBT TGC	30.0[c] (−1.0)	27.5 (−3.5)	27.0 (−4.0)	28.0 (−3.0)
ON2	5′-dGTG TBB TGC	39.0[c] (+4.0)	36.5 (+2.8)	30.5 (−0.2)	30.0 (−0.5)
ON3	5′-dGTG BBB TGC	46.0[c] (+5.0)	45.5 (+4.8)	34.5 (+1.2)	34.0 (+1.0)
ON4	5′-dGTG BBB BGC	55.5[c] (+6.1)	56.0 (+6.2)	42.0 (+2.7)	42.5 (+2.9)
Mismatch sequences[d]					
ON4	5′-dGTG BBB BGC 3′-rCAC ACA ACG	33.5 (−22.0)	33.0 (−23.0)	27.0 (−15.0)	22.0 (−20.5)
ON4	5′-dGTG BBB BGC 3′-rCAC AGA ACG	46.0[c] (−9.5)	46.5 (−9.5)	33.0 (−9.0)	30.0 (−12.5)
ON4	5′-dGTG BBB BGC 3′-rCAC AUA ACG	36.5 (−19.0)	37.0 (−19.0)	22.0 (−20.0)	20.0 (−22.5)

[a] Matched target sequence 5′-rGCA AAA CAC.
[b] See Table 1. For the unmodified reference duplex, T_m = 31.0 °C.
[c] Data taken from Ref. 4
[d] Mismatch studies, in brackets the changes in melting temperature as compared to the matched duplex **ON4**:RNA.

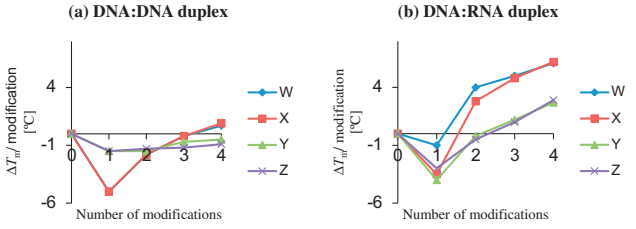


Figure 1. Graphical illustration of the hybridisation versus the number of modifications.

2.2. Hybridisation studies

The standard sequence chosen for the present study was a 9-mer oligonucleotide sequence studied previously.[3,4] with 1–4 consecutive incorporations of **W**, **X**, **Y** and **Z**. The oligonucleotides were mixed in medium salt buffer with the complementary DNA and RNA sequences and the melting temperatures (T_m) of the resulting duplexes were derived from the UV melting curves at neutral pH 7. Table 1 shows the results obtained with modified DNA:DNA duplexes. A single incorporation of either of the modified monomers **W**, **X**, **Y** or **Z** in the centre of the duplex, **ON1**, lead to a significant decrease in duplex stability. However, the decrease in thermal stability is less pronounced in the case of alkynyl monomers as validated by ΔT_m's of around −1.5 °C compared to the unmodified duplex in case of alkynyl monomers **Y** and **Z** as compared to ΔT_m's of around −5 °C in case of triazolyl monomers **Y** and **Z**. There was absolutely no influence whatsoever with respect to the position of sulfonamide group being *para* or *meta*. With the second incorporation of the modified monomers in **ON2**, the destabilizing influence in the case of triazolyl monomers **W** and **X** was reduced. In fact, a relative increase in duplex stability was observed when comparing **ON2** with **ON1**. On the other hand, the destabilizing influence of alkynyl monomers was found to be additive with ΔT_m's of around −1.5 for each of the two incorporations of **Y** or **Z**. In the case of triazolyl monomers **W** and **X**, the same trend continued with the three incorporations in **ON3**, whereby the destabilizing influence levelled off and the duplexes demonstrated unchanged thermal stability as compared to the unmodified duplex. With the four incorporations of **W** and **X** in **ON4**, the decrease in duplex stability was fully compensated by the stacking of the modified nucleobases. Hence the duplex stabilities were slightly increased in the case of both isomers **W** (ΔT_m = ~+0.7 °C per mod.) and **X** (ΔT_m = ~+0.9 °C per mod.). In the case of alkynyl monomers **Y** and **Z**, three or four consecutive incorporations virtually fail to evoke any further response, stabilizing or destabilizing. However, the *meta* isomer **Z** seems to be slightly more destabilizing than the *para* isomer **Y**. Figure 1a shows a graphic connection between ΔT_m and the number of modified moieties in the DNA:DNA duplex.

Table 2 shows the hybridisation data of the modified DNA:RNA duplexes. A single incorporation of either **W**, **X**, **Y** or **Z** in **ON1** leads to decreases in T_m that were somewhat smaller than with DNA:DNA in case of triazolyl monomers **W** and **X**, whereas the decrease in T_m was larger than with DNA:DNA in case of alkynylnucleoside monomers **Y** and **Z**. Surprisingly, a larger decrease in T_m (−3.5 °C) was observed with the *meta*-sulfonamido isomer of phenyltriazolylnucleoside monomer **X** while a smaller decrease in T_m (−1 °C) was observed with the *para*-sulfonamido isomer **W**. Incorporation of the second modified nucleoside in **ON2** leads to dramatically enhanced duplex stabilities in case of both triazolylnucleosides **W** and **X**, although the effect of the new *meta* isomer **X** is slightly smaller as compared to *para* isomer **W** (ΔT_m = +4.0 °C and +2.8 °C per modification, respectively). Overall, the introduction of the second phenyltriazole moiety on top of the first gave an identical increase in T_m of +9.0 °C in both cases (compare **ON2** with **ON1**). In comparison, the introduction of a second alkynylnucleoside monomer **Y** and **Z** gave increases in T_m of +3.5 °C and +2.0 °C, respectively, however, still maintains an overall slightly destabilized duplex as compared to the unmodified duplex.

The tendency to stabilize the duplex with each successive incorporation continued in general by three and four incorporations in **ON3** and **ON4** revealing further relative increases in duplex stability with ΔT_m's of +6.1 °C per modification for the four sulfonamide substituted phenyltriazoles in **W** and **X**, and ΔT_m's of +2.8 °C per modification for the four sulfonamide substituted phenylalkynyls in **Y** and **Z**. In other words, the forth incorporation of a triazole derivative increased the overall thermal stability of the duplex with around 10 °C (comparing **ON3** with **ON4**), whereas the forth incorporation of an alkynyl derivative increased the overall thermal stability of the duplex with around 7.5 °C. Overall, no differences whatsoever could be observed between *meta* and *para* isomers concerning relative duplex stabilities with three or four consecutive incorporations in **ON3** and **ON4**. Figure 1b demonstrates graphically how the ΔT_m evolves by the number of modifi-

cations clearly showing the deep decreases by one modification changing to large increases by consecutive incorporations.

Mismatch studies were also performed in order to further probe the RNA recognition. Hence, **ON4** with either **W**, **X**, **Y** or **Z** was mixed with RNA-sequences containing a single central mismatch, and the melting temperatures of the mismatched duplex were determined (Table 2). In all cases, fine mismatch discrimination was observed as indicated by the large decreases in T_m relative to the matched duplexes formed by **ON4**. A to C mismatches were perfectly discriminated by all the four modified nucleosides **W**, **X**, **Y** and **Z** (ΔT_m values >−15 °C). Similar results were observed for the A to U mismatches, whereas the discrimination of the A to G mismatches was slightly less efficient for all the four modifications **W**–**Z** with the largest discrimination attained by the *meta*-sulfonamidophenyl alkynylnucleoside monomer **Z** (ΔT_m = −12.5 °C). Overall, this is similar to unmodified DNA:RNA duplexes, where the A to G mismatch is the most stable of the three.

3. Discussion

In the present study, we have investigated the directional influence of the sulfonamide moiety in complementing the aromatic stacking in the major groove of nucleic acid duplexes. Hence, our recent results indicated a contribution of the sulfonamide moiety in improving the stacking efficiency of C5-phenyltriazolenucleosides.[4] At the same time, the study presents an opportunity to directly compare the stacking efficiency of the triazole and ethynyl linker in between the phenylsulfonamide and the nucleobase in the modified nucleoside.

As far as the optimum number of consecutive incorporations of modified C5-functionalized nucleosides is concerned, the hybridisation data for the oligonucleotides of the current study validates the trend of our first study demonstrating that one incorporation of any of the four modified nucleoside monomers leads to decreased stability of both DNA:DNA and DNA:RNA duplexes (Fig. 1). On the other hand, four consecutive incorporations demonstrate a massive increase in stability of the DNA:RNA duplex while having a net neutral effect on the stability of the DNA:DNA duplex. In the case of phenyltriazoles, two consecutive incorporations are enough to give most of the positive effect (Table 2 and Fig. 1). All the modified nucleosides demonstrate potent preferential recognition of RNA as compared to DNA while maintaining the capability of mismatch discrimination. In future antisense applications, the sequence design will be a balance between the RNA-affinity and overall sequence specificity and might be optimal with two consecutive incorporations.

Comparing the influence of substitution pattern in the phenyltriazole, the two isomeric sulfonamidonucleosides **W** and **X** exhibited almost similar effect on duplex stability indicating virtually non-existent influence of the position of sulfonamide on the phenyl ring. Similarly, no obvious influence of substitution pattern of sulfonamide group in C5-alkynylnucleosides was observed as there was only a slight difference in duplex stability of two modified nucleosides **Y** and **Z**.

A direct comparison of triazolyl and alkynyl nucleosides clearly demonstrates that the stacking efficiency of triazole group is significantly better than that of an alkynyl group (compare **ONs** having **W** or **X** with **Y** or **Z**) attaining a maximum increase in duplex stability of +6.1 °C per modification in case of C5-triazole modified nucleosides as compared to +2.9 °C per modification in case of C5-alkynyl modified nucleosides. This indicates that overall the triazole is a much more efficient moiety for obtaining a high RNA-affinity but also much more dependent on at least two consecutive incorporations. This might be due to a combination of the increased surface for π–π-stacking and the different orientation of the adjacent phenylgroups. Modelling has indicated an optimal positioning of these in the triazoles.[4,5]

The very similar hybridisation properties of **W** and **X** demonstrate that the efficient stacking is more dependent on the large aromatic surfaces of the combined phenyl and triazole rings than on the positioning of the sulfonamide. In other words, the organisation of neighbouring sulfonamide group, indicated by modelling on **ON4** containing **W**,[4] might be equally valid in the case of the *meta* substituted rings in **X**. This also indicates a large room for different substituents on the phenyltriazoles without compromising the high RNA-affinity.

In conclusion, phenyltriazole substituents on the 5-position of uracil with or without sulfonamides is a simple very easily synthesized tool for designing oligonucleotides with very high RNA-affinity for potential antisense therapeutics. The demand for two consecutive incorporations is complimented by the fact that also a phenyltriazole on the 5-position of cytosine can participate in the stacking and add to the increase in affinity.[5] We hope that these and other building blocks that are in progress can be significant tools in the development of therapeutic oligonucleotides.

4. Conclusions

Three new simple building blocks have been synthesized from the easily available 5-ethynyl-2′-deoxyuridine introducing alkynyl as well as triazole moieties into the major groove of nucleic acid duplexes. The stacking of sulfonamide substituted phenyltriazoles and phenylalkynyls in the major groove leads to very stable DNA:RNA duplexes. However, significantly more stable duplexes are attained with the C5-triazole modified nucleosides as compared to the C5-alkynyl modified nucleosides, and only two consecutive phenyltriazole moieties are necessary to give a pronounced effect. Hereby, the new building blocks can be promising tools in antisense research.

5. Experimental section

All commercial reagents were used as supplied, except CH_2Cl_2 which was distilled prior to use. Anhydrous solvents were dried over 4 Å activated molecular sieves (CH_2Cl_2, pyridine and DCE) or 3 Å activated molecular sieves (CH_3CN). Reactions were carried out under argon or nitrogen when anhydrous solvents were used. All mixtures of solvents were prepared as a volume to volume ratio (v/v). All reactions were monitored using TLC analysis with Merck silica gel plates (60 F_{254}). To visualize the plates, they were exposed to UV light (254 nm) and/or immersed in a solution of 5% H_2SO_4 in methanol (v/v) followed by charring. Column chromatography was performed with Silica Gel 60 (particle size 0.040–0.063 μm, Merck). NMR spectra were recorded on a Varian Gemini 2000 spectrometer or on Bruker Advance III 400/300 spectrometers. Values for δ are in ppm relative to tetramethylsilane as an internal standard or 85% H_3PO_4 as an external standard (^{31}P NMR). Assignments of NMR-signals when given are based on 2D spectra and follow standard nucleoside convention. High Resolution ESI mass spectra were recorded on a Thermo Finnigan TSQ 700 spectrometer, and EI mass spectra were recorded on a Finnigan SSQ 710 spectrometer. IR spectra were recorded either on ABB MB 3000 DTGS or on Perkin–Elmer 1720 Infrared Fourier Transform spectrometer.

5.1. Synthesis of 5-(1-(3-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4′-dimethoxytrityl)-2′-deoxyuridine (3)

To a solution of nucleoside **1** (300 mg, 0.53 mmol) and protected 3-azidobenzenesulfonamide **7b** (210 mg, 0.83 mmol) in

THF:H_2O:*t*-BuOH (10 mL, 3:1:1, v/v/v) was added aq sodium ascorbate (1 M, 1.2 mL, 1.2 mmol) and aq $CuSO_4$ (1.1 mL, 7.5%, w/v, 0.32 mmol). The resulting clear solution was stirred at rt for 6 h, and then diluted with EtOAc (30 mL) and brine (30 mL). The phases were separated, and the organic phase was washed with a saturated aqueous solution of $NaHCO_3$ (30 mL). The combined aqueous phase was extracted with EtOAc (2 × 30 mL), and the combined organic phase was dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by column chromatography (0–5% MeOH in CH_2Cl_2) to afford the nucleoside **3** (225 mg, 51%) as a pale yellow solid. R_f 0.4 (5% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.86 (br s, 1H, NH), 8.87 (s, 1H, HC=N)), 8.59 (s, 1H, triazole-H), 8.32 (s, 1H, Ar), 8.18 (s, 1H, H-6), 8.00 (d, 1H, *J* = 8.0 Hz, Ar), 7.95 (d, 1H, *J* = 8.0 Hz, Ar), 7.60 (t, 1H, *J* = 8.0 Hz, Ar), 7.41 (d, 1H, *J* = 8.0 Hz, Ar), 7.31–7.34 (m, 4H, Ar), 7.23 (t, 2H, *J* = 8.0 Hz, Ar), 7.11-7.15 (m, 2H, Ar), 6.80 (d, 2H, *J* = 8.0 Hz, Ar), 6.78 (d, 2H, *J* = 8.0 Hz, Ar), 6.43 (t, 1H, *J* = 8.0 Hz, H-1′), 4.44 (m, 1H, H-3′), 3.72 (s, 6H, 2 × OCH_3), 3.47 (dd, *J* = 10.2, 4.4 Hz, 1H, H-5′), 3.41 (dd, *J* = 10.2, 4.4 Hz, 1H, H-5′), 3.12 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 2.92 (d, ex, *J* = 8.0 Hz, 1H, 3′-OH), 2.57 (m, 1H, H-2′), 2.31–2.36 (m, 1H, H-2′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.3, 159.5, 158.4, 149.6, 144.6, 144.2, 139.9, 137.3, 136.8, 135.7, 135.6, 130.2, 130.1, 130.0, 128.1, 127.8, 126.8, 123.6, 120.2, 118.2, 113.2, 105.7, 86.8, 86.1, 85.8, 77.3, 77.0, 76.7, 72.4, 63.7, 55.2, 41.6, 40.6, 35.7. HiRes ESI MS *m/z* (M+Na) found/calcd 830.2554/830.2584.

5.2. Synthesis of 5-(1-(3-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4′-dimethoxytrityl)-3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*′-diisopropylaminophosphinyl)-2′-deoxyuridine (5)

The nucleoside **3** (135 mg, 0.16 mmol) was dried by the co-evaporation with anhydrous 1,2-dichloroethane (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5 mL). DIPEA (0.15 mL, 0.86 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropyl-phosphoramidochloridite (0.12 mL, 0.50 mmol) were added, and the reaction mixture was stirred at rt for 2 h whereupon it was quenched with 2–3 drops of absolute ethanol and evaporated to dryness. The resulting crude residue was purified by column chromatography (0–2% MeOH in CH_2Cl_2) to afford the phosphoramidite **5** (140 mg, 80%) as a white foam. R_f 0.4 (2% MeOH in CH_2Cl_2). ^{31}P NMR ($CDCl_3$, 162 MHz) δ 149.1, 148.7. HiRes ESI MS *m/z* (M+Na) found/calcd 1030.3617/1030.3663.

5.3. Synthesis of 3-azidobenzenesulfonamide (6b)

To an ice cold stirred solution of metanilamide[11] (1.0 g, 5.81 mmol) in 4 N HCl (50 mL) was added an aqueous $NaNO_2$ solution (0.48 g, 6.97 mmol in 3 mL H_2O) dropwise, maintaining the temperature of reaction mixture at 0–5 °C. To the diazotized mixture was then added a saturated aqueous solution of $NaHCO_3$ dropwise slowly to neutralize the reaction mixture (pH∼7.0), carefully maintaining the temperature below 5 °C. To this neutralized solution was added rapidly aqueous NaN_3 solution (1.13 g, 17.43 mmol in 5 mL H_2O) and the solution was stirred vigorously for 1 h. The reaction mixture was extracted with ethyl acetate (3 × 20 mL). The organic extract was washed with brine (2 × 20 mL), dried ($MgSO_4$) and concentrated under reduced pressure to afford the target azide **6b** (0.77 g, 67%) as an orange solid. The solid was washed with 25 mL petroleum ether, dried and used without further purification in the next step. R_f 0.6 (50% EtOAc in petroleum ether). Mp 127–128 °C. IR (KBr) cm^{-1} 3340 & 3263 (N-H stretch), 2114 (azide stretch), 1319 & 1173 (SO_2 stretch). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.65 (m, 1H, H-4), 7.58 (t, 1H, *J* = 1.92 Hz, H-2), 7.51 (t, 1H, *J* = 7.96 Hz, H-5), 7.33 (s, ex, 2H, SO_2NH_2), 7.22 (m, 1H, H-6); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 100 MHz): δ 145.6, 140.2, 130.0, 121.87, 121.86, 116.1.

5.4. Synthesis of *N*-dimethylaminomethylidine-3-azidobenzenesulfonamide (7b)

To a cold stirred solution of $POCl_3$ (0.48 mL, 5.05 mmol) in dimethylformamide (10 mL) was added 3-azidobenzenesulfonamide **6b** (500 mg, 2.52 mmol). The reaction mixture was stirred at rt for 3 h, and then poured into cold water and neutralized with saturated aqueous ammonia. The formed precipitates were isolated, washed with water (50 mL) and dried to afford the product **7b** (440 mg, 69%) as a white solid. R_f 0.3 (50% EtOAc in petroleum ether). Mp 136–138 °C. IR (KBr) cm^{-1} 2106 (azide stretch), 1628 (C=N stretch), 1335 & 1119 (SO_2 stretch). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.21 (s, 1H, CH=N), 7.58 (m, 1H, H-4), 7.51 (t, 1H, *J* = 7.84 Hz, H-5), 7.45 (t, 1H, *J* = 1.84 Hz, H-2), 7.2 (m, 1H, H-6), 3.20 (s, 3H, NCH_3), 2.99 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 100 MHz): δ 159.6, 144.3, 140.1, 130.1, 122.2, 121.8, 116.3, 41.0 (NCH_3), 35.0 (NCH_3).

5.5. Synthesis of *N*-dimethylaminomethylidine-4-iodobenzenesulfonamide (7c)

To a cold stirred solution of $POCl_3$ (1.95 mL, 21.20 mmol) in dimethylformamide (25 mL) was added 4-iodobenzenesulfonamide.[12] **6c** (3.0 g, 10.60 mmol). The reaction mixture was stirred at rt for 3 h, and then poured into cold water and neutralized with saturated aqueous ammonia. The formed precipitates were isolated, washed with water (200 mL) and dried to afford the product **7c** (2.33 g, 65%) as a light orange solid. R_f 0.3 (50% EtOAc in petroleum ether). Mp 172–174 °C. IR (KBr) cm^{-1} 1620 (C=N stretch), 1142 & 1342 (SO_2 stretch). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.20 (s, 1H, CH=N), 7.91 (d, 2H, *J* = 8.0 Hz, H-2 & 6), 7.53 (d, 2H, *J* = 8.0 Hz, H-3 & 5), 3.14 (s, 3H, NCH_3), 2.90 (s, 3H, NCH_3);^{13}C NMR (DMSO-d_6, 100 MHz) δ 159.8, 142.6, 137.7, 127.6, 99.3, 40.8 (NCH_3), 35.0 (NCH_3).

5.6. Synthesis of *N*-dimethylaminomethylidine-3-iodobenzenesulfonamide (7d)

To a cold stirred solution of $POCl_3$ (0.82 mL, 8.83 mmol) in dimethylformamide (15 mL) was added 3-iodobenzenesulfonamide.[13] **6d** (1.25 g, 4.41 mmol). The reaction mixture was stirred at rt for 3 h, and then poured into cold water and neutralized with saturated aqueous ammonia. The formed precipitates were isolated, washed with water (150 mL) and dried to afford the product **7d** (1.22 g, 82%) as white solid. R_f 0.3 (50% EtOAc in petroleum ether). Mp 140–143 °C. IR (KBr) cm^{-1} 1620 (C=N stretch), 1142 & 1335 (SO_2 stretch). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.22 (s, 1H, CH=N), 8.05 (s, 1H, H-2), 7.93 (d, 1H, *J* = 7.8 Hz, H-6), 7.78 (d, 2H, *J* = 7.8 Hz, H-4), 7.33 (t, 1H, *J* = 7.8 Hz, H-5), 3.14 (s, 3H, NCH_3), 2.90 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$/DMSO-d_6, 100 MHz) δ 159.5, 144.3, 140.0, 134.1, 130.3, 125.1, 93.8, 41.1 (NCH_3), 35.1 (NCH_3).

5.7. Synthesis of 5-(4-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)ethynyl-5′-(4,4′-dimethoxytrityl)-2′-deoxyuridine (8)

The nucleoside **1** (550 mg, 1.0 mmol), protected 4-iodobenzenesulfonamide **7c** (500 mg, 1.5 mmol), CuI (40 mg, 0.2 mmol) and $Pd(PPh_3)_4$ (120 mg, 0.1 mmol) were suspended in anhydrous DMF (10 mL) and the contents were degassed and placed under an argon atmosphere while stirring in the dark for 30 min at rt. Anhydrous Et_3N (0.6 mL, 4.25 mmol) was added and the mixture was stirred

overnight in the dark at rt under argon atmosphere. After reaction completion, the mixture was concentrated under reduced pressure and added EtOAc (70 mL) and saturated aqueous solution of $NaHCO_3$ (50 mL). The phases were separated, and the organic phase was washed with brine (50 mL). The combined aqueous phase was extracted with EtOAc (40 mL), and the combined organic phase was dried (Na_2SO_4), and concentrated under reduced pressure to get the crude product which was purified by column chromatography (0–5% MeOH in CH_2Cl_2) to afford the nucleoside **8** (500 mg, 65%) as a white foam. R_f 0.4 (5% MeOH in CH_2Cl_2). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.47 (br s, 1H, NH), 8.33 (s, 1H, HC=N), 8.09 (s, 1H, H-6), 7.61 (d, 2H, J = 8.0 Hz, Ar), 7.43–7.45 (m, 2H, Ar), 7.31–7.34 (m, 4H, Ar), 7.24–7.28 (m, 2H, Ar), 7.14 (m, 1H, Ar), 6.94 (d, 2H, J = 8.0 Hz, Ar), 6.77 (d, 2H, J = 8.0 Hz, Ar), 6.75 (d, 2H,, J = 8.0 Hz, Ar), 6.36 (t, 1H, J = 8.0 Hz, H-1′), 4.57 (m, 1H, H-3′), 4.12 (m, 1H, H-4′), 3.69 (s, 6H, 2 × OCH_3), 3.53 (dd, J = 10.2, 4.4 Hz, 1H, H-5′), 3.29 (dd, J = 10.2, 4.4 Hz, 1H, H-5′), 3.13 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 2.53 (m, 1H, H-2′), 2.35 (m, 1H, H-2′), 2.16 (br s, 1H, 3′-OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.4, 159.3, 158.8, 149.3, 144.5, 143.0, 142.4, 135.6, 135.5, 131.8, 130.0, 128.3, 128.2, 128.0, 127.2, 126.3, 126.1, 113.5, 100.1, 92.5, 87.2, 86.8, 85.9, 83.0, 72.3, 63.5, 55.3, 41.9, 41.6, 35.7. HiRes ESI MS m/z (M+Na) found/calcd 787.2398/787.2408.

5.8. Synthesis of 5- (3-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)ethynyl-5′-(4,4′-dimethoxytrityl)-2′-deoxyuridine (9)

The nucleoside **1** (550 mg, 1.0 mmol), protected 3-iodobenzenesulfonamide **7d** (500 mg, 1.5 mmol), CuI (40 mg, 0.2 mmol) and $Pd(PPh_3)_4$ (120 mg, 0.1 mmol) were suspended in anhydrous DMF (10 mL) and the mixture was degassed and placed under an argon atmosphere while stirring in the dark for 30 min at rt. Anhydrous Et_3N (0.6 mL, 4.25 mmol) was added and the mixture was stirred overnight in the dark at rt under argon atmosphere. After reaction completion, the mixture was concentrated under reduced pressure and added EtOAc (70 mL) and saturated aqueous solution of $NaHCO_3$ (50 mL). The phases were separated, and the organic phase was washed with brine (50 mL). The combined aqueous phase was extracted with EtOAc (40 mL), and the combined organic phase was dried (Na_2SO_4), and concentrated under reduced pressure to get the crude product which was purified by column chromatography (0–5% MeOH in CH_2Cl_2) to afford the nucleoside **9** (500 mg, 65%) as a white foam. R_f 0.4 (5% MeOH in CH_2Cl_2). ^1H NMR ($CDCl_3$, 400 MHz) δ 9.17 (br s, 1H, NH), 8.23 (s, 1H, HC=N), 8.08 (s, 1H, H-6), 7.77–7.80 (m, 2H, Ar), 7.42–7.43 (m, 2H, Ar), 7.32–7.35 (m, 4H, Ar), 7.18–7.26 (m, 4H, Ar), 7.14 (m, 1H, Ar), 6.78 (d, 2H, J = 8.0 Hz, Ar), 6.76 (d, 2H, J = 8.0 Hz, Ar), 6.36 (t, 1H, J = 8.0 Hz, H-1′), 4.58 (m, 1H, H-3′), 4.14 (m, 1H, H-4′), 3.67 (s, 6H, 2 × OCH_3), 3.43 (dd, J = 10.2, 4.4 Hz, 1H, H-5′), 3.35 (dd, J = 10.2, 4.4 Hz, 1H, H-5′), 3.13 (s, 3H, CH_3), 2.96 (s, 3H, CH_3), 2.92 (br s, 1H, 3′-OH), 2.7 (m, 1H, H-2′), 2.34 (m, 1H, H-2′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.3, 159.3, 158.6, 149.1, 144.4, 142.8, 142.4, 135.5, 129.98, 129.91, 129.3, 128.3, 128.0, 127.8, 127.0, 123.3, 113.3, 99.9, 92.2, 87.1, 86.7, 86.0, 81.5, 72.3, 63.5, 55.2, 41.7, 41.5, 35.5. HiRes ESI MS m/z (M+Na) found/calcd 787.2382/787.2408.

5.9. Synthesis of 5-(4-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)ethynyl-5′-(4,4′-dimethoxytrityl)-3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*′-diisopropylaminophosphinyl)-2′-deoxyuridine (10)

The nucleoside **8** (200 mg, 0.26 mmol) was dried by the co-evaporation with anhydrous 1,2-dichloroethane (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5 mL). DIPEA (0.30 mL, 1.72 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropylphosphoramidochloridite (0.20 mL, 0.84 mmol) were added, and the reaction mixture was stirred at rt for 2 h whereupon it was quenched with 2–3 drops of absolute ethanol and evaporated to dryness. The resulting crude residue was purified by column chromatography (0–2.5% MeOH in CH_2Cl_2) to afford the phosphoramidite **10** (170 mg, 67%) as a white foam. R_f 0.4 (2% MeOH in CH_2Cl_2). ^{31}P NMR ($CDCl_3$, 162 MHz) δ 149.1, 148.6. HiRes ESI MS m/z (M+Na) found/calcd 987.3514/987.3492.

5.10. Synthesis of 5-(3-(*N*-((dimethylamino)methylidene) aminosulfonyl)phenyl)ethynyl-5′-(4,4′-dimethoxytrityl)-3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*′-diisopropylaminophosphinyl)-2′-deoxyuridine (11)

The nucleoside **9** (200 mg, 0.26 mmol) was dried by the co-evaporation with anhydrous 1,2-dichloroethane (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5 mL). DIPEA (0.30 mL, 1.72 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropylphosphoramidochloridite (0.20 mL, 0.84 mmol) were added, and the reaction mixture was stirred at rt for 2 h whereupon it was quenched with 2–3 drops of absolute ethanol and evaporated to dryness. The resulting crude residue was purified by column chromatography (0–2.5% MeOH in CH_2Cl_2) to afford the phosphoramidite **11** (185 mg, 73%) as a white foam. R_f 0.4 (2% MeOH in CH_2Cl_2). ^{31}P NMR ($CDCl_3$, 162 MHz) δ 149.0, 148.6. HiRes ESI MS m/z (M+Na) found/calcd 987.3453/987.3492.

5.11. Synthesis of oligodeoxynucleotides

Oligonucleotide synthesis was carried out on an automated DNA synthesizer following the phosphoramidite approach. Synthesis of oligonucleotides **ON1**–**ON4** (**W**–**Z**) was performed on a 0.2 μmol scale by using the amidites **4**, **5**, **10** and **11** as well as the corresponding commercial 2-cyanoethyl phosphoramidites of the natural 2′-deoxynucleosides. The synthesis followed the regular protocol for the DNA synthesizer. For compound **4**, **5**, **10** and **11**, a prolonged coupling time of 15 min was used. 1*H*-Tetrazole was used as the activator and coupling yields for all 2-cyanoethyl phosphoramidites were 95–99.8%. The 5′-*O*-DMT-ON oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at 55 °C for 16 h, which also removed the protecting groups. The oligonucleotides were purified by reversed-phase HPLC on a Waters 600 system using a X_{terra} prep MS C_{18}; 10 μm; 7.8×150 mm column; Buffer A: 0.05 M Triethyl Ammonium Acetate pH 7.4. Buffer B: MeCN/H_2O (1:1). Program used: 2 min 100% A, 100–30% A over 38 min, 10 min 100% B, 10 min 100% A. All oligonucleotides were detritylated by treatment with an 80% aqueous solution of acetic acid for 30 min, quenched with an aqueous solution of sodium acetate (3 M, 15 μL) and then added sodium perchlorate (5 M, 15 μL) followed by acetone (1 mL). The pure oligonucleotides precipitated over night at −20 °C. After centrifugation 12000 rpm, 10 min at 4 °C, the supernatant was removed, and the pellet washed with cold acetone (2 × 1 mL) and dried for 30 min under reduced pressure, and dissolved in pure water (1000 μL). The concentration was determined by UV at 260 nm, and the purity confirmed by IC analysis. MALDI-TOF-MS $[M-H]^-$ gave the following results (calcd/found): **ON1-X** (2943.6/2941.3); **ON2-X** (3151.4/3151.1); **ON3-X** (3359.2/3361.4); **ON4-X** (3567.0/3570.9); **ON1-Y** (2900.6/2900.9); **ON2-Y** (3065.4/3065.8); **ON3-Y** (3230.2/3229.8); **ON4-Y** (3395.0/3398.4); **ON1-Z** (2900.6/2901.6); **ON2-Z** (3065.4/3067.6); **ON3-Z** (3230.2/3231.5); **ON4-Z** (3395.0/3397.4);

6. Thermal denaturation experiments

UV melting experiments were carried out on a UV spectrometer. Samples were dissolved in a medium salt buffer containing 2.5 mM

Na_2HPO_4, 5 mM NaH_2PO_4, 100 mM NaCl, and 0.1 mM EDTA at pH = 7.0 with 1.5 μM concentrations of the two complementary sequences. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 5 to 75 °C at a rate of 1.0 °C/min by means of a Peltier temperature programmer. The melting temperature was determined as the local maximum of the first derivatives of the absorbance vs. temperature curve. The melting curves were found to be reversible. All determinations are averages of at least duplicates within ± 0.5 °C.

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