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Convergent synthesis and properties of photoactivable NADPH mimics targeting nitric oxide synthases†

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A new series of photoactivable NADPH mimics bearing one or two *O*-carboxymethyl groups on the adenosine moiety have been readily synthesized using click chemistry. These compounds display interesting one- or two-photon absorption properties. Their fluorescence emission wavelength and quantum yields (Φ) are dependent on the solvent polarity, with a red-shift in a more polar environment ($\lambda_{max,em}$ = 460–467 nm, Φ > 0.53 in DMSO, and $\lambda_{max,em}$ = 475–491 nm, Φ < 0.17 in Tris). These compounds show good binding affinity towards the constitutive nNOS and eNOS, confirming for the first time that the carboxymethyl group can be used as a surrogate of phosphate. Two-photon fluorescence imaging of nanotriggers in living cells showed that the presence of one carboxymethyl group (especially on the 3′ position of the ribose) strongly favors the addressing of nanotriggers to eNOS in the cell context.

Introduction

Nitric oxide (NO) is involved in numerous biological processes, from neurotransmission, hormone secretion, platelet aggregation and adhesion to vasodilation, the immune response, infection and cancer.[1] NO synthase (NOS) is a heme- and flavin-containing enzyme that catalyzes the synthesis of NO through two serial monooxygenase reactions. Electrons are transferred from NADPH, through the flavins FAD and FMN, to the heme iron, where molecular oxygen is activated, allowing formation of NO and L-citrulline from L-arginine. There are three isoforms in mammals: neuronal NOS (nNOS) and endothelial NOS (eNOS) which are constitutive and modulated by intracellular Ca^{2+}/calmodulin (CaM), and inducible NOS (iNOS). nNOS and eNOS control neuron function and blood pressure, respectively, while iNOS is associated with the immune response. NOSs are functional dimers, with each monomer containing an N-terminal oxygenase domain with binding sites for arginine and tetrahydrobiopterin, and a reductase domain with binding sites for the flavins and NADPH, connected by a linker containing a Ca^{2+}/calmodulin binding site.[2] The biological effect of NO is highly dependent on its concentration, site and dosage.[3] Various strategies have been developed for therapeutic proposes based on lowering (through inhibition of NOS by targeting the oxygenase or the reductase domain)[4] or enhancing the concentration of NO (with NO-donors),[5] or by combining NO-donors with drugs.[6] A light-activated reaction should allow a spatiotemporal modulation of NO concentration. In this context, both photoactive NO donors[7] and caged NOS inhibitors[8] have been recently developed for various biomedical applications.

Directly targeting the NADPH binding site of NOS with a NADPH analog whose redox potential can be modulated upon light absorption is another way to photocontrol the biosynthesis of NO. Replacing the nicotinamide moiety of NADPH by dienyl chromophore substituted by two donor groups had led to a photoactivable molecule (nanotrigger NT1 Fig. 1) designed to fit in the NADPH site of nNOS that is triggered by one- or two-photon excitation to donate electrons synchronously to nNOS or eNOS, resulting in NO formation.[9] By linking the adenosine nucleotide to a push–pull 4-(4-nitrostyryl)anilinyl chromophore, which is unable to provide the electrons required for NOS catalysis, a NOS inhibitor (nanoshutter NS1, Fig. 1) has been generated that also imaged eNOS upon two-photon excitation.[10] These interesting results led to the elaboration of a straightforward route for the synthesis of new suitable nanotriggers for time-resolved studies of the NOS catalytic mechanism. Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition (CuAAC)

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†Electronic supplementary information (ESI) available: Absorption and fluorescence titration spectra, immuno-staining of eNOS on fixed cells, ^{1}H and ^{13}C NMR spectra of all compounds. See DOI: 10.1039/c6ob01533f

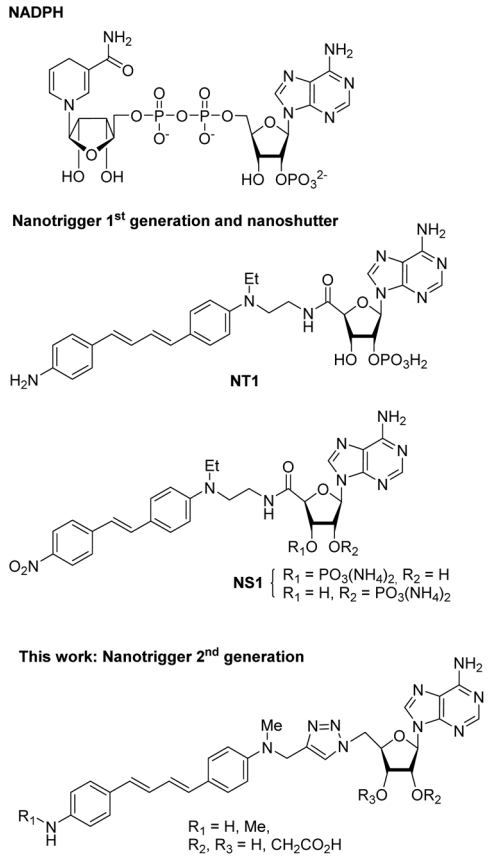


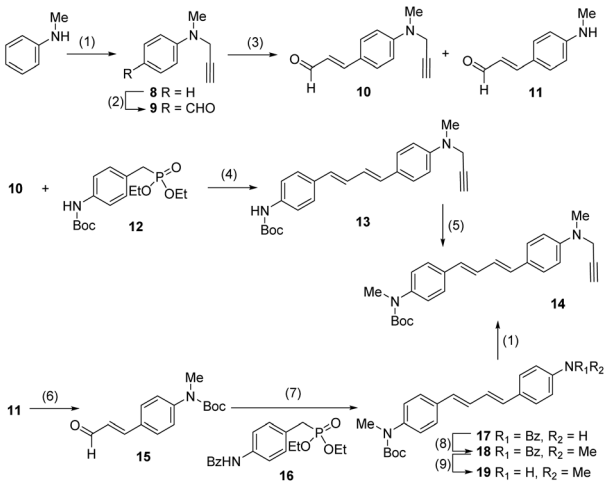
Fig. 1 Structure of NADPH and its photoactivable mimics.

has been proven versatile for the generation of libraries of bioactive compounds including nucleoside and nucleotide derivatives,[11] as the formed 1,2,3-triazole ring is resistant to hydrolysis, oxidation and reduction. We have designed a novel generation of photoactivable NADPH mimics with three modifications: (a), replacement of the 2′-phosphate by a carboxymethyl group on the 2′-, 3′- or both the 2′- and 3′-positions to determine whether the carboxymethyl group could be used as a surrogate for phosphate and to study the influence of the substitution position on its recognition by NOS, since the phosphate group on the 2′-position of NADPH is in equilibrium with 3′-*O*-phosphate in solution; (b), introduction of a methyl group on the terminal amino function to investigate its impact on NT photophysical properties in comparison with the amine as a donor group; (c), use a triazole for linking the photoactive chromophore moiety with the adenosine moiety targeting the NADPH recognition site (Fig. 1).

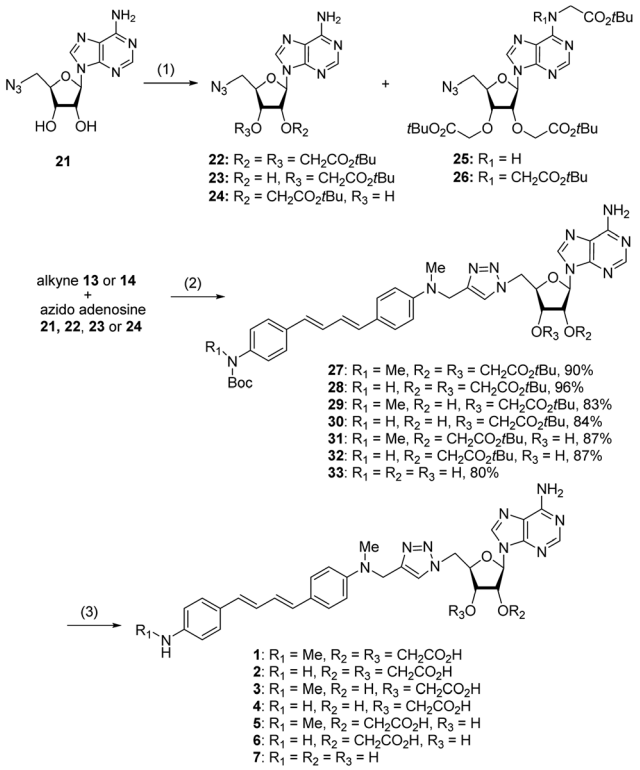
Results and discussion

Synthesis of photoactivable NADPH mimics

The second generation of nanotriggers was then synthesized by CuAAC between the alkyne-functionalized dienyl chromophore **13** or **14** (Scheme 1) and 5′-azido adenosine derivatives **22–24** (Scheme 2). The chromophore **13** was prepared from the *N*-methylaniline, through *N*-propargylation and formylation reactions to afford aldehyde **9** (Scheme 1). Wittig reaction of **9**



Scheme 1 Synthesis of chromophores **13** and **14**. (1) Propargyl bromide, K_2CO_3, MeCN, 95% for **8**; 52% for **14** (from **19**). (2) $POCl_3$, DMF, 90 °C, 82%. (3) i. tributyl(1,3-dioxolan-2-ylmethyl)phosphonium bromide, NaH, THF, 0 °C to r.t.; ii. AcOH–H_2O, CH_2Cl_2, 60% **10**, 20% **11**. (4) NaH, THF, 45%. (5) MeI, NaH, THF, 94%. (6) Boc_2O, THF, reflux, 90%. (7) NaH, THF, 56%. (8) MeI, NaH, THF, 90%. (9) NaOH, EtOH, reflux, 76%.



Scheme 2 Synthesis of photoactivable NADPH mimics **1–7**. (1) $BrCH_2CO_2tBu$, NaH, DMF, −45 °C to r.t. (2) $CuSO_4{\cdot}5H_2O$, Na ascorbate, DMF–H_2O. (3) AcCl, MeOH.

with tributyl[(1,3-dioxolan-2-yl)methyl]phosphonium bromide gave, after mild acidic hydrolysis, the desired α,β-unsaturated aldehyde **10** (60%) as well as the depropargylated compound **11** (20%) which might be formed by hydrolysis of ynamine, the rearrangement product of *N*-propargyl amine in the presence of NaH.[12] Horner–Emmons–Wadsworth reaction of aldehyde **10** with the phosphonate **12** afforded the diene **13** which was *N*-methylated to give the compound **14**. The diene **14** could also be prepared from the aldehyde **11** through *N*-Boc protection to α,β-unsaturated aldehyde **15** followed by reaction with the phosphonate **16**, *N*-methylation and removal of benzoyl group. The Boc-protecting group in **13** could be readily removed by AcCl in methanol to deprotected dienyl chromophore **20**. *O*-Alkylation of 5′-azido adenosine **21**[13] with NaH and *tert*-butyl α-bromoacetate led to a mixture of *O*- and *N*-alkylated products **22–26**, depending on the quantity of reagents (Scheme 2). Optimization with 1.1 equiv. NaH and 1 equiv. $BrCH_2CO_2tBu$ gave **22** (18%), **23** (18%) and **24** (6%), separable by column chromatography. The position of *O*-alkylation in **23** and **24** has been determined by comparison with ^{13}C NMR data in the literature.[14,15] Then, click reaction of alkyne **13** or **14** with azido adenosines **21–24** afforded compounds **27–33** in good yields. The target compounds **1–7** were readily obtained after acidic deprotection.

Photophysical studies

The absorbance and fluorescence properties of compounds **1–7** were next characterized in two different solvents: DMSO and Tris buffer (20 mM, pH 7.4). All NTs show similar absorption (Fig. S1†) and emission spectra (Fig. 2), and the main parameters are summarized in Table 1. For all NTs, the maximum wavelength for one-photon absorption ($\lambda_{max,abs}$) was found to be strongly dependent on the solvent condition, with a systematic red-shifted $\lambda_{max,abs}$ in DMSO compared to Tris (about 20 nm). Moreover, in each solvent, NTs with the electron donating group NHMe (compounds **1**, **3**, **5**) are characterized by a slight but significant red-shifted $\lambda_{max,abs}$ compared to other NT compounds having the electron donating group NH_2 (compounds **2**, **4**, **6**, **7**), with $\lambda_{max,abs}$ = 391–392 nm and 387–388 nm, respectively, in DMSO (370–372 nm and 367–368 nm, respectively, in Tris). This is consistent with the expected lower HOMO–LUMO energy gap with NHMe as a better electron donating group than NH_2. Regarding the molar extinction coefficients (ε), systematically higher ε values are observed in Tris as compared to DMSO for compounds **1** and **3** while the opposite tendency is noticed for compounds **2**, **4**, **6** and **7**, suggesting a differential interaction between the electron donor group and the solvent. To note, it has previously been shown that the electron accepting nitro group of NS1 at this terminal position is involved in probing solvation in its direct vicinity.[10] The influence of the solvent or the electron donating group was much less pronounced on the optimal wavelength for two-photon absorption ($\lambda_{max,2\text{-}PE}$ = 750 nm, regardless of the compound or the solvent), with however a higher two-photon absorption cross-section (σ^2) in DMSO compared to Tris. Except for compound **7**, the σ^2 values (in the 41–68 GM range in DMSO) of NTs remain compatible with the characteristic values found for NS1[10] or NT1.[9] The fluorescence emission wavelength ($\lambda_{max,em}$) was dependent on the solvent with an expected red-shift in a more polar environment ($\lambda_{max,em}$ = 460–467 nm in DMSO depending on the compound and 475–491 nm in Tris). For all NT compounds, the fluorescence quantum yields (Φ) were dramatically different in Tris buffer and DMSO (Φ < 0.17 and Φ > 0.53, respectively), suggesting that the binding of NTs to NOS should be associated with a substantial NT fluorescence enhancement (confirmed thereafter). As found for one-photon absorption parameters, fluorescence emission of the NTs is dependent on the nature of the electron donating group, especially their Φ values, with the NHMe group leading to higher Φ values than NH_2 (Φ = 0.84–0.91 and 0.53–0.79, respectively, in DMSO).

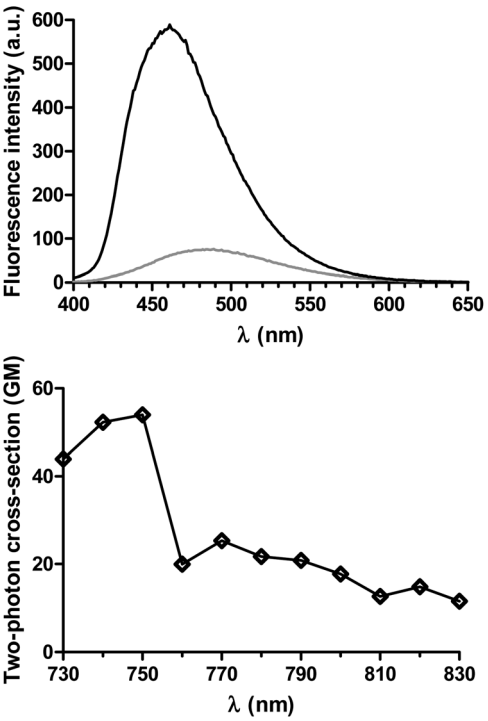


Fig. 2 Representative fluorescence emission and two-photon excitation spectra of compound **3** (20 μM). Top, fluorescence emission spectra in Tris buffer (grey) or DMSO (black). Excitation wavelengths were 370 and 390 nm in Tris and DMSO, respectively. Excitation and emission slits were 5 nm and the PMT (photomultiplier tube) voltage was 600 V. Bottom, two-photon excitation spectrum in DMSO (laser power, 50 mW).

Binding assay

The apparent binding affinities of the NTs with nNOS and eNOS were evaluated by the increase in the NT intrinsic fluorescence at 460 nm (excitation 420 nm) that was blue shifted upon binding to protein, as previously reported with NT1[9d] (Fig. S2,† Table 2).[16] Compounds **1** to **6** bound to both nNOS and eNOS, showing slightly greater affinity to eNOS over nNOS.

Table 1 Photophysical properties of compounds **1–7**

	$\lambda_{max,abs}$, $\lambda_{max,1\text{-PE}}$ [a] (nm)		Molar extinction coefficient (ε) [b] (cm^{-1} M^{-1})		$\lambda_{max,2\text{-PE}}$ [c] (nm)		Two-photon absorption cross section (σ^2) [d] (GM)		$\lambda_{max,em}$ (nm)		Quantum yield (ϕ) [e]		2-$\hbar\nu$ brightness ($\sigma^2 \times \phi$) [d,e] (GM)	
	DMSO	Tris	DMSO	Tris	DMSO	Tris	DMSO	Tris	DMSO	Tris	DMSO	Tris	DMSO	Tris
1	392	372	43 100 ± 1700	53 300 ± 2800	750	750	53	11.1	464	488	0.91	0.09	48	1
2	388	367	70 800 ± 700	54 600 ± 1200	750	750	68	28.2	460	475	0.79	0.17	54	4.8
3	391	370	50 000 ± 3500	81 500 ± 1100	750	750	58	8.3	467	490	0.89	0.06	52	0.5
4	387	367	79 400 ± 2700	37 800 ± 1600	750	750	41	14.2	463	479	0.56	0.07	23	1
5	391	372	81 300 ± 2900	nd [f]	750	750	51	nd	467	491	0.84	nd	43	nd
6	388	367	84 600 ± 2500	66 000 ± 1200	750	750	47	11.4	463	476	0.60	0.07	28	0.8
7	387	368	63 700 ± 1000	37 600 ± 800	750	750	28	10	461	478	0.53	0.03	15	0.3

[a] 1-PE: one-photon excitation. [b] The reported data are the mean of n = 3–4 experiments. [c] 2-PE: two-photon excitation. [d] 1 Göppert-Mayer = 10^{-50} cm^4 s per photon. [e] SD (standard deviations) are typically 10–15% (n = 2–3) for fluorescence quantum yield and two-photon brightness values. [f] nd: non-determined for stability reason.[16]

Table 2 App K_d values in μM (±standard error) for **1–7** with nNOS and eNOS

	nNOS	eNOS
1	38.3 ± 3.1	19.5 ± 1.1
2	21.5 ± 0.7	14.7 ± 1.1
3	26.8 ± 2.1	19.5 ± 1.1
4	32.6 ± 2.7	28.6 ± 4.6
6	30.0 ± 3.8	24.1 ± 1.9
7	58.0 ± 14.5	>80
NT1[9a]	7 ± 3	
NS1[10]	4.3 ± 0.6	

Compound **1** bearing two carboxymethyl groups shows the greatest selectivity for eNOS over nNOS. Although the binding affinities of compounds **1–6** were lower than those of NT1 or NS1, these results confirmed that the carboxymethyl group could be used as a surrogate of phosphate. Compound **7** without carboxymethyl group bound poorly to nNOS and very poorly to eNOS. Furthermore, no significant difference exists between mono- or di-*O*-carboxymethyl compounds (**3**,**4**,**6** *vs.* **1**,**2**), between 2- or 3-*O*-carboxymethyl derivatives (**6** *vs.* **3**,**4**), and amino- or *N*-methylamino derivatives (**2**,**4**,**6** *vs.* **1**,**3**). Nevertheless, an effect of charge on binding to eNOS is observed in that the apparent affinity of compounds **2** > **4** ≈ **6** ≫ **7**.

Cellular imaging studies

Thanks to relatively good σ^2 and Φ, the two-photon fluorescence brightnesses of the 2nd-generation NTs with values spanning from 15 to 54 GM are compatible with imaging studies using multiphoton microscopy. Taking into account the significant fluorescence enhancement of NT compounds when they are bound to eNOS, we next addressed the question of NT fluorescence brightness in the cell context. Two-photon fluorescence imaging of NTs in living cells was then performed in two distinct cell lines, endothelial HUVEC cells (Human Umbilical Vein Endothelial Cells) and HeLa cells (cervical, cancer) that express and do not express eNOS, respectively.[17–20] In a previous study, we confirmed that HUVECs efficiently express eNOS by using immuno-staining of eNOS.[10] Here, by using the same immuno-staining procedure, we confirmed that no expression of eNOS occurs in HeLa cells (Fig. S3†). HUVEC and HeLa cells were incubated with the different NT compounds for varying times and imaged using λ_{ex} = 760 nm (Fig. 3). No detectable NT fluorescence was obtained in HeLa cells whereas, in sharp contrast, a two-photon emission is observed in HUVECs, however to different extent depending on the compound. Among the different compounds, **3** and **4** displayed the highest intracellular fluorescence intensities in HUVECs and were characterized by a rapid cellular uptake (less than 5 min). Weaker fluorescence intensities were detected with **1** and **7** and very weak or no signal was detected with **2** and **6**. This hierarchy (**3**,**4** ≫ **1**,**7** > **2**,**6**) is clearly different than the hierarchy established for two-photon brightness values as

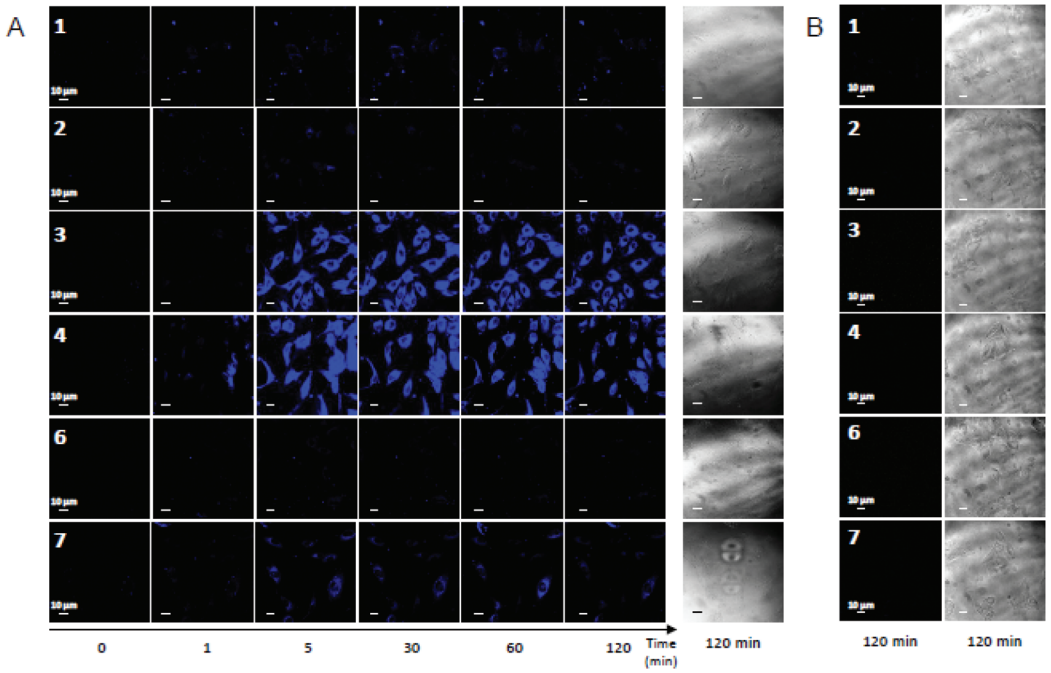


Fig. 3 Two-photon fluorescence images of NT compounds (**1–4**, **6**, **7**) in living cells. HUVEC (A) or HeLa (B) cells were treated for varying times with NT compounds (final concentration, 5 μM). Compound **5** was not studied for stability reason (see text). Imaging was performed at fixed time intervals (explicitly mentioned below the images). Excitation was 760 nm and the emission slit setting was 450–550 nm. Only fluorescence images corresponding to t = 120 min are shown for HeLa cells and only DIC (differential interference contrast) transmission images corresponding to t = 120 min are shown for HUVEC and HeLa cells.

derived from *in vitro* experiments (**2** > **3** > **1** > **6** > **4** > **7** in Table 1). Note that compound **5** was discarded from the imaging study because of its particular chemical instability in culture medium. The differential fluorescence emission in HUVEC and HeLa cells correlates well with the expression level of eNOS. The imaging study shows that the presence of one carboxymethyl group (especially on the 3′ position of the ribose as in compounds **3** and **4**) strongly favors the addressing of NTs to eNOS in the cell context. Finally, the similar behavior of compounds **3** and **4** indicates that the nature of the electron donating group on the chromophore (NH_2 *vs.* NHMe) does not play an important role for eNOS addressing.

Conclusions

In summary, we have synthesized a series of photoactive NADPH mimics bearing one or two *O*-carboxymethyl groups on the adenosine moiety. These compounds display good binding affinity towards the constitutive nNOS and eNOS, confirming that the carboxymethyl group can be used as a surrogate of phosphate. Two-photon fluorescence imaging of NTs in living cells showed a two-photon emission in HUVECs, with the highest intracellular fluorescence intensities displayed by compounds **3** and **4** bearing one carboxymethyl group on the 3′ position of the ribose, while no detectable NT fluorescence was obtained in HeLa cells.

Experimental

General

All commercial available reagents purchased from Sigma-Aldrich, TCI Chemical and Carlo-Erba were used without further purification. Column chromatography was performed on Silica gel 60 (40–60 μm). The solvents for column chromatography were used without purification. The reactions carried out under anhydrous conditions are performed under argon in glassware previously dried in an oven. Methanol is dried over molecular sieve 3 Å. THF, DMF, dichloromethane, acetonitrile and toluene were previously dried through alumina cartridge using a solvent purificator MBRAUN SPS-800. Reactions were monitored by TLC on Silica Gel 60F-254 plates with detection by UV (254 nm or 365 nm) or by spraying with 10% H_2SO_4 in EtOH and heating about 30 s at 400–600 °C. Melting points were determined with a Kofler melting point apparatus. Optical rotations were measured using a Jasco P-2000 polarmeter at room temperature in a 10 cm, 1 mL cell. NMR spectra were recorded on a JEOL ESC-400 spectrometer in $CDCl_3$, CD_3OD or DMSO-d_6 solution. Chemical shift was given in units of parts per millon related

to TMS or solvent protons as internal reference. High-resolution mass Spectra (HRMS) were recorded on a Q-TOF MaXis using standard conditions or Bruker Microflex™ MALDI-TOF mass spectrometry.

General procedure for the deprotection of Boc

AcCl (40 equiv.) was added slowly to MeOH at 0 °C under Ar. After stirring 5 min at 0 °C, the Boc-protected substrate was added. The solvent was evapored and the residue co-evapored with MeOH (3×) after completion of reaction (2 h).

N-Methyl-*N*-(prop-2-yn-1-yl)aniline (8)

To a une solution of *N*-methylaniline (1.66 mL, 15.2 mmol) in acetonitrile (30 mL) at 0 °C under Ar, were added K_2CO_3 (3.154 g, 22.8 mmol) and propargyl bromide (80% in toluene, 2.54 mL, 22.8 mmol) drop by drop. After one night reaction at r.t., the mixture was filtrated on silica gel and the solvent evapored under reduced pressure. The residue was then purified by chromatography on silica gel (petroleum ether/AcOEt: 50/1) to afford the title compound as an orange oil (2.101 g, 95%). R_f = 0.30 (petroleum ether/AcOEt: 50/1); IR (ATR): 3289, 1675, 1599, 1504, 1337, 1243, 1199, 1114, 997, 754, 691 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.29–7.25 (m, $2H_{ar}$), 6.88–6.80 (m, $3H_{ar}$), 4.05 (d, 2H, J = 2.3 Hz, CH_2), 2.98 (s, 3H, CH_3), 2.17 (t, 1H, J = 2.3 Hz, CH≡); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 149.0 (C_q), 129.2 (CH_{ar}), 118.4, 114.3 (CH_{ar}); 79.4 (C_q), 72.1 (CH), 42.5 (CH_2), 38.6 (CH_3).

4-[*N*-Methyl-*N*-(prop-2-yn-1-yl)amino]benzaldehyde (9)

To dry DMF (150 mL) under Ar at 0 °C, was added dropwise $POCl_3$ (4.10 mL, 43.9 mmol). After 10 min stirring at 0 °C and 15 min at r.t., a solution of **8** (6.371 g, 43.88 mmol) in dry DMF (10 mL) was added dropwise at 0 °C. The reaction mixture was stirred 1 h at r.t. and 4 h at 90 °C. After neutralization with aqueous saturated solution of $NaHCO_3$, the solvent was evaporated and the residue extracted with CH_2Cl_2 (3 × 500 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 9/1.5) to give the aldehyde **9** as a yellow solid (6.258 g, 82%). R_f = 0.33 (petroleum ether/AcOEt: 9/1.5); mp: 74–76 °C; IR (ATR): 3246, 2821, 2748, 1651, 1591, 1550, 1533, 1385, 1335, 1239, 1165, 998, 937, 816, 740, 680 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 9.76 (s, 1H, CHO), 7.77–7.74 (m, $2H_{ar}$), 6.82–6.80 (m, $2H_{ar}$), 4.12 (d, 2H, J = 2.3 Hz, CH_2), 3.08 (s, 3H, CH_3), 2.24 (t, 1H, J = 2.3 Hz, CH≡); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 190.5 (CHO), 153.2 (C_q), 131.9 (CH_{ar}), 126.5 (C_q), 112.3 (CH_{ar}), 78.3 (C_q), 72.6 (CH), 41.8 (CH_2), 38.4 (CH_3); HRMS (ESI) m/z: Calcd for $C_{11}H_{12}NO$ $[M + H]^+$: 174.0919; Found: 174.0908.

(2*E*)-3-{4-[*N*-Methyl-*N*-(prop-2-yn-1-yl)amino]phenyl}prop-2-enal (10) and (2*E*)-3-[4-(N-methylamino)phenyl]prop-2-enal) (11)

To a solution of **9** (869 mg, 5.00 mmol) in dry THF (25 mL) under Ar at 0 °C, were added tributyl(1,3-dioxolan-2-ylmethyl) phosphonium bromide (2.52 g, 6.51 mmol) and NaH (60%, 600 mg, 15.0 mmol). After one night reaction at r.t., the reaction mixture was neutralized with a saturated solution of NH_4Cl. After solvent evaporation, the residue was extraited with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. The obtained acetal was then dissolved in CH_2Cl_2 (25 mL) and treated with a mixture of CH_3CO_2H/H_2O (0.15 mL/0.75 mL) during 2 days at r.t., neutralised with a saturated solution of $NaHCO_3$. After solvent evaporation, the residue was extracted with AcOEt (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (CH_2Cl_2/AcOEt: 10/0.1) to afford the title compound **10** as an orange solid (598 mg; 60%) and the depropargylated compound **11** as a red brown powder (173 mg; 20%).

Compound **10**: R_f = 0.39 (CH_2Cl_2/AcOEt: 10/0.1); mp: 89–91 °C; IR (ATR): 3318, 1644, 1582, 1530, 1388, 1138, 958, 805 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 9.60 (d, 1H, J = 7.8 Hz, CHO), 7.49–7.47 (m, $2H_{ar}$), 7.39 (d, 1H, J = 15.6 Hz, CH═), 6.82–6.80 (m, $2H_{ar}$), 6.57 (dd, 1H, J = 7.8, 15.6 Hz, CH═), 4.11 (d, 2H, J = 2.3 Hz, CH_2), 3.07 (s, 3H, CH_3), 2.24 (t, 1H, J = 2.3 Hz, CH≡); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 193.9 (CHO), 153.6 (CH), 151.1 (C_q), 130.5 (CH_{ar}), 124.8 (CH), 123.5 (C_q), 113.3 (CH_{ar}), 78.6 (C_q), 72.5 (CH), 41.9 (CH_2), 38.5 (CH_3); HRMS (ESI) m/z: Calcd for $C_{13}H_{14}NO$ $[M + H]^+$: 200.1075; Found: 200.1068.

Compound **11**: R_f = 0.27 (CH_2Cl_2/AcOEt: 10/0.1); mp: 111–113 °C; IR (ATR): 3215, 1644, 1582, 1519, 1378, 1327, 1135, 927, 806, 730 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 9.59 (d, 1H, J = 7.8 Hz, CHO), 7.43–7.41 (m, $2H_{ar}$), 7.37 (d, 1H, J = 15.6 Hz, CH═), 6.60–6.58 (m, $2H_{ar}$), 6.54 (dd, 1H, J = 15.6, 7.8 Hz, CH═), 2.90 (s, 3H, CH_3); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 193.9 (CHO), 154.4 (CH), 152.2 (C_q), 130.7 (CH_{ar}), 123.4 (CH), 122.4 (C_q), 112.0 (CH_{ar}), 30.0 (CH_3); HRMS (ESI) m/z: Calcd for $C_{10}H_{12}NO$ $[M + H]^+$: 162.0919; Found: 162.0910.

Diethyl 4-*tert*-butoxycarbonylamino-benzylphosphonate (12)

To a solution of diethyl 4-aminobenzylphosphonate (487 mg, 2.00 mmol) in dry THF (3.5 mL) under Ar, was added Boc_2O (1.00 g, 4.58 mmol). After stirring at reflux during 4 h, the solvent was evaporated and a saturated solution of NH_4Cl added. The aqueous layer was then extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 1/4) to give **12** as a white solid (672 mg, 98%). R_f = 0.31 (petroleum ether/AcOEt: 1/4); mp: 116–118 °C; IR (ATR): 3273, 2979, 1709, 1533, 1417, 1242, 1162, 1053, 1031, 965 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.34–7.32 (m, $2H_{ar}$), 7.21–7.18 (m, $2H_{ar}$), 6.94 (br s, 1H, NH), 4.05–3.95 (m, 4H, $2CH_2$), 3.10 (d, 2H, J = 21.1 Hz, CH_2), 1.51 (s, 9H, *t*Bu), 1.24 (t, 6H, J = 6.9 Hz, $2CH_3$); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 152.9, 137.5 (C_q); 130.3 (CH_{ar}), 125.8 (d, J = 8.0 Hz, C_q), 118.7 (CH_{ar}), 80.4 (C_q), 62.2 (CH_2), 33.0 (d, J = 137 Hz, CH_2), 28.4, 16.4 (CH_3); HRMS (ESI) m/z: Calcd for $C_{16}H_{27}NO_5P$ $[M + H]^+$: 344.1627; Found: 344.1623.

4-[(1*E*,3*E*)-4-(4-*tert*-Butoxycarbonylaminophenyl)buta-1,3-dienyl]-*N*-methyl-*N*-(prop-2-ynyl)aniline (13)

To a suspension of NaH (60% in oil, 2.271 g, 56.78 mmol) in dry THF (15 mL) under Ar at 0 °C, were added dropwise a solution of **12** (6.842 g, 18.93 mmol) in dry THF (20 mL) and a solution of **10** (1.875 g, 9.46 mmol) in dry THF (20 mL). After stirring 6 h at r.t., the reaction mixture was neutralised with a saturated solution of NH_4Cl. After concentration, the residue was extracted with CH_2Cl_2 (3 × 250 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/CH_2Cl_2: 1/4) to afford **13** as a yellow solid (1.654 g, 45%). R_f = 0.42 (petroleum ether/CH_2Cl_2: 1/4); mp: 179–181 °C; IR (ATR): 3365, 3262, 1701, 1516, 1235, 1162, 1110, 978, 799 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.35–7.25 (m, $6H_{ar}$), 6.88–6.74 (m, 4H, $2H_{ar}$, 2CH═), 6.59–6.51 (m, 2H, 2CH═), 4.05 (d, 2H, J = 2.3 Hz, CH_2), 2.99 (s, 3H, CH_3), 2.18 (t, 1H, J = 2.3 Hz, CH≡), 1.52 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 152.7, 148.5, 137.4, 132.9 (C_q); 132.4, 130.4, 128.8 (CH); 127.8 (C_q), 127.5, 126.9 (CH_{ar}); 126.3 (CH), 118.7, 114.2 (CH_{ar}); 80.7, 79.2 (C_q); 72.2 (CH), 42.4 (CH_2), 38.7, 28.5 (CH_3); HRMS (ESI) m/z: Calcd for $C_{25}H_{29}N_2O_2$ $[M + H]^+$: 389.2229; Found: 389.2222.

***N*-Methyl-4-[(1*E*,3*E*)-4-(4-*tert*-butoxycarbonylaminophenyl) buta-1,3-dienyl]-*N*-methyl-*N*-(prop-2-ynyl)aniline (14)**

From **13**: To a solution of **13** (530 mg, 1.37 mmol) in dry THF (7 mL) under Ar at 0 °C, was added NaH (60% in oil, 218 mg, 5.46 mmol) and MeI (170 μL, 2.73 mmol). After stirring 6 h at r.t., the reaction mixture was neutralised with a saturated solution of NH_4Cl. After concentration, the residue was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/CH_2Cl_2: 1/4) to afford **14** as a yellow solid (514 mg, 94%).

From **19**: To a solution of **19** (77 mg, 0.21 mmol) in acetonitrile (5 mL) under Ar at 0 °C, was added K_2CO_3 (116 mg, 0.84 mmol) and propargyl bromide (80% in toluene, 94 μL, 0.844 mmol) drop by drop. After one night stirring at r.t., the mixture was filtrated on silica gel and the solvent evapored under reduced pressure. The residue was then purified by chromatography on silica gel (petroleum ether/AcOEt: 4/1) to afford **14** (44 mg, 52%). R_f = 0.42 (petroleum ether/CH_2Cl_2: 1/4); mp: 155–157 °C; IR (ATR): 3234, 2976, 1685, 1602, 1509, 1476, 1431, 1365, 1255, 1152, 1109, 989, 850, 803 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.36 (d, 2H, J = 8.2 Hz, $2H_{ar}$), 7.33 (d, 2H, J = 8.2 Hz, $2H_{ar}$), 7.16 (d, $2H_{ar}$, J = 8.2 Hz, $2H_{ar}$), 6.91–6.74 (m, 4H, $2H_{ar}$, 2CH), 6.58 (d, 1H, J = 15.1 Hz, CH═), 6.53 (d, 1H, J = 15.1 Hz, CH═), 4.02 (d, 2H, J = 2.3 Hz, CH_2), 3.23 (m, 3H, CH_3), 2.96 (s, 3H, CH_3), 2.17 (t, 1H, J = 2.3 Hz, CH≡), 1.45 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 154.7, 148.5, 142.6, 134.8 (C_q); 132.9, 130.1, 129.7 (CH); 127.5 (CH_{ar}), 127.5 (C_q), 126.2 (CH_{ar}), 126.0 (CH), 125.5, 114.1 (CH_{ar}); 80.4, 79.2 (C_q); 72.2 (CH), 42.3 (CH_2), 38.6, 37.2, 28.4 (CH_3); HRMS (ESI) m/z: Calcd for $C_{26}H_{31}N_2O_2$ $[M + H]^+$: 403.2385; Found: 403.2380.

(2*E*)-3-{4-[*N*-Methyl-*N*-(*tert*-butoxycarbonyl)amino]phenyl}prop-2-enal (15)

To a solution of **11** (646 mg, 4.02 mmol) in dry THF (80 mL) under Ar at 0 °C, was added Boc_2O (3.509 g, 16.08 mmol). After refluxing during 48 h, the solvent was evaporated. Dichloromethane and a saturated solution of NH_4Cl were then added. After layers separation, the aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 4/1) to afford **15** as a white solid (950 mg, 90%). R_f = 0.35 (petroleum ether/AcOEt: 4/1); mp: 59–61 °C; IR (ATR): 2974, 2053, 1703, 1669, 1601, 1511, 1430, 1354, 1256, 1148, 1126, 1106, 969, 813 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 9.68 (d, 1H, J = 7.8 Hz, CHO), 7.54 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.46 (d, 1H, J = 15.6 Hz, CH═), 7.35 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 6.68 (dd, 1H, J = 7.8, 15.6 Hz, CH═), 3.30 (s, 3H, CH_3), 1.49 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 193.8 (CHO), 154.2 (C_q), 152.2 (CH═), 146.4, 130.5 (C_q); 128.8 (CH_{ar}), 128.0 (CH═), 125.2 (CH_{ar}), 81.1 (C_q), 36.9, 28.3 (CH_3). HRMS (ESI) m/z: Calcd for $C_{15}H_{20}NO_3$ $[M + H]^+$: 262.1438; Found: 262.1437.

Diethyl 4-benzoylamino-benzylphosphonate (16)

To a solution of diethyl 4-aminobenzylphosphonate (1.95 g, 8.01 mmol) in toluene (80 mL), were added benzoyl chloride (1.02 mL, 8.81 mmol) and Et_3N (1.67 mL, 12.02 mmol). After one night reaction at r.t., water was added. The aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 1/4) to afford **16** as a white solid (2.75 g, 99%). R_f = 0.16 (petroleum ether/AcOEt: 1/4); mp: 157–159 °C; IR (ATR): 3271, 3245, 2985, 1651, 1601, 1532, 1515, 1414, 1323, 1246, 1057, 972, 843, 704 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.60 (s, 1H, NH), 7.93–7.90 (m, $2H_{ar}$), 7.65–7.62 (m, $2H_{ar}$), 7.55–7.42 (m, $3H_{ar}$), 7.27–7.22 (m, $2H_{ar}$), 3.99–3.94 (m, 4H, $2CH_2$), 3.12 (d, 2H, J = 21.6 Hz, CH_2), 1.24–1.20 (m, 6H, $2CH_3$); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 166.1 (C_q), 137.4 (C_q), 131.8, 130.4, 130.3, 128.7, 127.4 (CH_{ar}); 127.2 (d, J = 10 Hz, C_q), 120.6 (CH_{ar}), 62.3 (CH_2), 33.2 (d, J = 138 Hz, CH_2), 16.5 (CH_3); HRMS (ESI) m/z: Calcd for $C_{18}H_{22}NO_4P$ $[M + H]^+$: 348.1365; Found: 348.1363.

4-[(1*E*,3*E*)-4-(Benzoylaminophenyl)buta-1,3-dienyl]-*N*-methyl-*N*-(*tert*-butoxycarbonyl)aniline (17)

To a suspension of NaH (60% in oil, 576 mg, 14.4 mmol) in dry THF (20 mL) under Ar at 0 °C, were added dropwise a solution of **16** (1.876 g, 5.401 mmol) in dry THF (10 mL) and a solution of **15** (942 mg, 3.60 mmol) in dry THF (20 mL). After stirring 6 h at r.t., the reaction mixture was neutralised with a saturated solution of NH_4Cl. After concentration, the residue was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (CH_2Cl_2/AcOEt: 1/0.05) to afford **17** as a yellow solid (914 mg, 56%). R_f = 0.61 (CH_2Cl_2/AcOEt: 1/0.05); mp: 212–214 °C; IR (ATR): 3326, 2926,

1700, 1646, 1601, 1587, 1514, 1411, 1365, 1321, 1254, 1153, 1110, 984, 854, 804 cm^{-1}; RMN ^1H (DMSO-d_6, 400 MHz): δ 10.32 (s, 1H, NH), 7.98–7.94 (m, $2H_{ar}$), 7.80 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.62–7.46 (m, $7H_{ar}$), 7.25 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.10–7.00 (m, 2H, 2CH═), 6.73–6.68 (m, 2H, 2CH═), 3.18 (s, 3H, CH_3), 1.41 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 165.7, 154.8, 143.2, 137.4, 135.1, 134.6, 133.9 (C_q); 132.1, 132.0, 129.2 (CH═); 129.0, 128.8, 127.2, 127.1, 126.6, 125.6, 120.4 (CH_{ar}); 80.6 (C_q), 37.3, 28.5 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{29}H_{30}N_2O_3$ $[M + H]^+$: 455.5680; Found: 455.5682.

4-[(1*E*,3*E*)-4-(*N*-Methyl-*N*-benzoylaminophenyl)buta-1,3-dienyl]-*N*-methyl-*N*-(*tert*-butoxycarbonyl)aniline (18)

To a solution of **17** (48 mg, 0.11 mmol) in dry THF (2 mL) under Ar at 0 °C, was added NaH (60% in oil, 13 mg, 0.33 mmol) and MeI (14 μL, 0.22 mmol). After stirring one night at r.t., the reaction mixture was neutralised with a saturated solution of NH_4Cl. After concentration, the residue was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 4/1) to afford **18** as a yellow solid (48 mg, 90%). R_f = 0.25 (petroleum ether/AcOEt: 4/1); mp: 74–76 °C; IR (ATR): 1694, 1643, 1600, 1512, 1364, 1283, 1152, 1106, 972, 840, 700 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.38–7.18 (m, $11H_{ar}$), 6.98 (d, 2H, J = 8.2 Hz, $2H_{ar}$), 6.88–6.84 (m, 2H, 2CH═), 6.64–6.53 (m, 2H, 2CH═), 3.50 (s, 3H, CH_3), 3.26 (s, 3H, CH_3), 1.46 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 170.5, 154.7, 144.0, 143.2, 136.0, 135.6, 134.2 (C_q); 132.7, 131.4, 129.9, 129.8 (CH═); 128.8, 128.7, 127.9, 127.0, 126.6, 125.5 (CH_{ar}); 80.6 (C_q), 38.4, 37.3, 28.4 (CH_3). HRMS (ESI) *m*/*z*: Calcd for $C_{30}H_{33}N_2O_3$ $[M + H]^+$: 469.2486; Found: 469.2488.

4-[(1*E*,3*E*)-4-(*N*-Methylaminophenyl)buta-1,3-dienyl]-*N*-methyl-*N*-(*tert*-butoxycarbonyl)aniline (19)

To a solution of **18** (1.356 g; 2.89 mmol) in EtOH (40 mL) at r.t., was added NaOH (4 M, 72 mL, 289 mmol). The reaction mixture was stirred at reflux during 8 h and 48h at r.t. After solvent evaporation, the residue was extracted with CH_2Cl_2 (3 × 80 mL). The combined organic layers were dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (petroleum ether/AcOEt: 4/1) to afford **19** as a red solid (766 mg, 73%). R_f = 0.33 (petroleum ether/AcOEt: 4/1); mp: 182–184 °C; IR (ATR): 3398, 2974, 2929, 1691, 1603, 1512, 1365, 1258, 1153, 1109, 987, 851, 804 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 7.34 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.30 (d, $2H_{ar}$, J = 8.3 Hz, $2H_{ar}$), 7.18 (d, $2H_{ar}$, J = 8.3 Hz, $2H_{ar}$), 6.90 (dd, 1H, J = 10.5, 15.1 Hz, CH═), 6.77 (dd, 1H, J = 10.5, 15.1 Hz, CH═), 6.62–6.52 (m, 4H, $2CH_{ar}$, 2CH═), 3.87 (s, 1H, NH), 3.30 (s, 3H, CH_3), 2.85 (s, 3H, CH_3), 1.47 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 154.8, 149.1, 142.6, 135.0 (C_q); 133.4, 129.9, 129.7 (CH═); 127.8 (CH_{ar}), 126.8 (C_q), 126.2, 125.5 (CH_{ar}); 125.2 (CH═), 112.5 (CH_{ar}), 80.4 (C_q), 37.3, 30.7, 28.5 (CH_3). HRMS (ESI) *m*/*z*: Calcd for $C_{23}H_{29}N_2O$ $[M + H]^+$: 365.2224; Found: 365.2224.

4-[(1*E*,3*E*)-4-(4-Aminophenyl)buta-1,3-dien-1-yl]-*N*-methyl-*N*-(prop-2-yn-1-yl)aniline (20)

Boc deprotection of compound **13** (131 mg, 0.337 mmol) according to the general procedure afforded **20** (99 mg, 100%) as a solid. mp: 188–190 °C; IR (ATR): 3394, 2967, 2920, 2878, 2584, 1596, 1508, 1465, 986, 853 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 7.66 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.63 (d, 2H, J = 8.3 Hz, $2H_{ar}$), 7.57 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.37 (d, 2H, J = 8.7 Hz, $2H_{ar}$), 7.14–7.10 (m, 2H, 2CH═), 6.81–6.77 (m, 2H, 2CH═), 4.49 (d, 2H, J = 2.3 Hz, CH_2), 3.31 (s, 3H, CH_3), 3.20 (m, 1H, CH≡); RMN ^{13}C (CD_3OD, 100 MHz): δ 141.3, 140.1, 139.8 (C_q); 133.3, 132.9, 132.2, 131.9 (CH); 130.7 (C_q), 129.0, 124.4, 122.3 (CH_{ar}); 80.6 (C_q), 74.0 (CH), 49.4 (CH_2), 44.0 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{20}H_{20}N_2O$ $[M + H]^+$: 289.1705; Found: 289.1708.

O-Alkylation of 5′-azido-5′-deoxy-adenosine (21)

To a suspension of NaH (60% in oil, 159 mg, 3.98 mmol) in dry DMF (18 mL) at 0 °C, was added in portion compound **21** (1.054 g, 3.607 mmol) under Ar. The reaction mixture was stirred at 0 °C for 2 h, then cooled to −45 °C, to which *tert*-butyl bromoacetate (587 μL, 3.98 mmol) was added slowly. After one night reaction at r.t., the reaction mixture was neutralised with a saturated solution of NH_4Cl. After solvent evaporation, the residue was extracted with AcOEt (3 × 100 mL). The combined organic layers were washed with saturated NH_4Cl (3 × 200 mL), dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.5) to afford to 5′-azido-5′-deoxy-2′,3′-di-*O*-(*tert*-butoxycarbonylmethyl)-adenosine (**22**) (360 mg, 18%), 5′-azido-5′-deoxy-2′-*O*-(*tert*-butoxycarbonylmethyl)-adenosine (**23**) (269 mg, 18%) and 5′-azido-5′-deoxy-3′-*O*-(*tert*-butoxycarbonylmethyl)-adenosine (**24**) (94 mg, 6%) as white solids. By using NaH (60% in oil, 117 mg, 2.92 mmol, 2 equiv.) in DMF (8 mL), **21** (427 mg, 1.46 mmol), and *tert*-butyl bromoacetate (431 μL, 2.92 mmol, 2 equiv.), compounds **22** (265 mg, 35%), **25** (5′-azido-5′-deoxy-2′,3′-di-*O*-(*tert*-butoxycarbonylmethyl)-N^6,N^6-di-(*tert*-butoxycarbonylmethyl)-adenosine) (142 mg, 13%) and **26** (5′-azido-5′-deoxy-2′,3′-di-*O*-(*tert*-butoxycarbonylmethyl)-N^6-(*tert*-butoxycarbonylmethyl)-adenosine) (74 mg, 8%) were obtained as white solids.

22: R_f = 0.24 (CH_2Cl_2/EtOH: 10/0.5); mp: 48–50 °C; $[\alpha]_D$ +53.4 (*c* 1.0, MeOH); IR (ATR): 3331, 3176, 2979, 2935, 2103, 1742, 1641, 1368, 1235, 1145, 848 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.30 (s, 1H, H_2), 8.20 (s, 1H, H_8), 6.19 (d, 1H, J = 4.6 Hz, $H_{1'}$), 4.97 (dd, 1H, J = 4.6, 5.0 Hz, $H_{2'}$), 4.47 (dd, 1H, J = 5.0, 5.5 Hz, $H_{3'}$), 4.34–4.32 (m, 1H, $H_{4'}$), 4.29–4.27 (m, 2H, CH_2), 4.24–4.22 (m, 2H, CH_2), 3.72–3.70 (m, 2H, $H_{5'}$), 1.49 (s, 9H, *t*Bu), 1.37 (s, 9H, *t*Bu); RMN ^{13}C (CD_3OD, 100 MHz): δ 171.3, 170.9 (C_q); 157.3 (C_6), 153.9 (C_2), 150.5 (C_4), 141.7 (C_8), 120.7 (C_5), 89.0 ($C_{1'}$), 82.9 (C_q), 82.8 ($C_{4'}$), 81.4 ($C_{2'}$), 79.2 ($C_{3'}$), 69.0 (CH_2), 52.8 ($C_{5'}$), 28.4, 28.3 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{22}H_{33}N_8O_7$ $[M + H]^+$: 521.2472; Found: 521.2463.

23: R_f = 0.07 (CH_2Cl_2/EtOH: 10/0.5); mp: 65–67 °C; $[\alpha]_D$ +51.9 (*c* 1.0, MeOH); IR (ATR): 3342, 3178, 2977, 2103, 1737,

1641, 1599, 1368, 1243, 1139, 798 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.29 (s, 1H, H_2), 8.20 (s, 1H, H_8), 6.05 (d, 1H, J = 5.0 Hz, $H_{1'}$), 4.91 (dd, 1H, J = 5.0, 4.6 Hz, $H_{2'}$), 4.34–4.33 (m, 1H, $H_{4'}$), 4.30 (t, 1H, J = 5.0 Hz, $H_{3'}$), 4.26–4.25 (m, 2H, CH_2), 3.70–3.69 (m, 2H, $H_{5'}$), 1.47 (s, 9H, *t*Bu); RMN ^{13}C (CD_3OD, 100 MHz): δ 171.9 (C_q), 157.3 (C_6), 153.9 (C_2), 150.6 (C_4), 141.3 (C_8), 120.5 (C_5), 90.2 ($C_{1'}$), 83.3 (C_q), 82.9 ($C_{4'}$), 81.0 ($C_{3'}$), 74.2 ($C_{2'}$), 69.5 (CH_2), 53.0 ($C_{5'}$), 28.3 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{23}N_8O_5$ $[M + H]^+$: 407.1791; Found: 407.1786.

24: R_f = 0.13 (CH_2Cl_2/EtOH: 10/0.5); mp: 64–66 °C; $[\alpha]_D$ +40.1 (*c* 1.0, MeOH); IR (ATR): 3438, 3334, 3180, 2980, 2930, 2102, 1737, 1641, 1247, 1140, 1082, 723 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.30 (s, 1H, H_2), 8.22 (s, 1H, H_8), 6.19 (d, 1H, J = 5.0 Hz, $H_{1'}$), 4.80 (t, 1H, J = 5.0 Hz, $H_{2'}$), 4.50 (dd, 1H, J = 5.0, 4.9 Hz, $H_{3'}$), 4.22–4.21 (m, 2H, CH_2), 4.20–4.17 (m, 1H, $H_{4'}$), 3.71–3.57 (m, 2H, $H_{5'}$), 1.37 (s, 9H, *t*Bu); RMN ^{13}C (CD_3OD, 100 MHz): δ 171.6 (C_q), 157.3 (C_6), 153.9 (C_2), 150.6 (C_4), 141.6 (C_8), 120.6 (C_q), 88.7 ($C_{1'}$), 84.5 ($C_{4'}$), 83.3 ($C_{2'}$), 83.2 (C_q), 71.5 ($C_{3'}$), 69.6 (CH_2), 52.9 ($C_{5'}$), 28.2 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{16}H_{23}N_8O_5$ $[M + H]^+$: 407.1791; Found: 407.1783.

25: R_f = 0.64 (CH_2Cl_2/AcOEt/EtOH: 2/1/0.1); mp: 48–50 °C; $[\alpha]_D$ +50.9 (*c* 1.0, $CHCl_3$); IR (ATR): 2979, 2932, 2103, 1741, 1621, 1585, 1369, 1230, 1148, 1090, 848 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.29 (s, 1H, H_2), 8.02 (s, 1H, H_8), 6.51 (br s, 1H, NH), 6.12 (d, 1H, J = 3.2 Hz, $H_{1'}$), 4.77–4.76 (m, 1H, $H_{2'}$), 4.37–4.15 (m, 8H, $H_{3'}$, $H_{4'}$, $3CH_2$), 3.78 (dd, 1H, J = 3.0, 13.3 Hz, $H_{5'}$), 3.70 (dd, 1H, J = 4.6, 13.3 Hz, $H_{5'}$), 1.44 (s, 27H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 169.4, 169.2 (C_q); 154.5 (C_6), 152.8 (C_2), 148.8 (C_4), 139.2 (C_8), 120.7 (C_q), 88.3 ($C_{1'}$), 82.1, 82.0 (C_q); 80.7 ($C_{4'}$), 80.2 ($C_{2'}$), 77.6 ($C_{3'}$), 68.0 (CH_2), 51.7 ($C_{5'}$), 28.2 (CH_3), 28.1 (CH_3).

26: R_f = 0.94 (CH_2Cl_2/AcOEt/EtOH: 2/1/0.1); mp: 29–31 °C; $[\alpha]_D$ +26.1 (*c* 1.0, $CHCl_3$); IR (ATR): 2976, 2925, 2103, 1741, 1590, 1368, 1226, 1149, 994, 848 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.27 (s, 1H, H_2), 8.00 (s, 1H, H_8), 6.12 (d, 1H, J = 2.3 Hz, $H_{1'}$), 5.00–4.89 (m, 2H, CH_2), 4.68–4.67 (m, 1H, $H_{2'}$), 4.57–4.07 (m, 8H, $H_{3'}$, $H_{4'}$, $3CH_2$), 3.83 (dd, 1H, J = 2.5, 13.0 Hz, $H_{5'}$), 3.73 (dd, 1H, J = 3.9, 13.0 Hz, $H_{5'}$), 1.46–1.41 (m, 36H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 169.5, 169.4, 169.3, 169.2 (C_q); 154.5 (C_6), 152.1 (C_2), 150.2 (C_4), 137.8 (C_8), 121.1 (C_q), 88.4 ($C_{1'}$), 82.0 (C_q), 81.8 (C_q), 80.5 ($C_{4'}$), 80.2 ($C_{2'}$), 77.4 ($C_{3'}$), 67.9 (CH_2), 67.8 (CH_2), 53.1 (CH_2), 51.7 ($C_{5'}$), 51.3 (CH_2), 28.2 (CH_3).

General procedure for CuAAC

To a solution of alkyne (1 equiv.) and azide (1 equiv.) in a mixture of DMF/H_2O (1/0.1, 2.2 mL $mmol^{-1}$ alkyne) at r.t. under Ar, were added $CuSO_4{\cdot}5H_2O$ (0.4 equiv.) and Na ascorbate (0.4 equiv.). After completion of reaction (4 h at r.t.), the solvent was removed *in vacuo* and the residue diluted in AcOEt and brine. The aqueous layer was extracted with AcOEt (2×). The combined organic layers were washed with brine (3×), dried over $MgSO_4$, filtered, evaporated and purified by column chromatography over silica gel to afford the triazole.

1-[5′-Deoxy-2′,3′-di-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl-*N*-*tert*-butoxycarbonyl) amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (27)

From alkyne **14** (212 mg, 0.523 mmol) and azido adenosine **22** (272 mg, 0.523 mmol), compound **27** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.4) as a yellow solid (434 mg, 90%). R_f = 0.34 (CH_2Cl_2/EtOH: 10/0.4); mp: 112–114 °C; $[\alpha]_D$ +10.5 (*c* 1.0, $CHCl_3$); IR (ATR): 2978, 1741, 1692, 1640, 1632, 1510, 1367, 1238, 1147, 984, 845, 732 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.19 (s, 1H, H_2), 7.72 (s, 1H, H_8), 7.35 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.24 (d, 2H, J = 8.4 Hz, $2H_{ar}$), 7.20 (s, 1H, CH═), 7.16 (d, 2H, J = 8.4 Hz, $2H_{ar}$), 6.86 (dd, 1H, J = 10.4, 15.2 Hz, CH═), 6.72 (dd, 1H, J = 10.4, 15.2 Hz, CH═), 6.57 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 6.53 (d, 2H, J = 15.2 Hz, 2CH═), 6.10 (d, 1H, J = 2.8 Hz, $H_{1'}$), 5.87 (br s, 2H, NH_2), 4.75–4.74 (m, 2H, CH_2), 4.71 (dd, 1H, J = 2.8, 4.4 Hz, $H_{2'}$), 4.55–4.42 (m, 4H, $H_{3'}$, $H_{4'}$, $2H_{5'}$), 4.34–4.17 (m, 2H, CH_2), 3.25 (s, 3H, CH_3), 2.90 (s, 3H, CH_3), 1.48 (s, 9H, *t*Bu), 1.45 (s, 9H, *t*Bu), 1.38 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 169.6, 169.5, 155.6, 154.8 (C_q); 153.0 (C_2), 149.3, 148.5, 145.2, 142.7 (C_q); 140.4 (C_8), 135.0 (C_q), 133.0, 129.9 (CH═); 127.7 (CH_{ar}), 126.4 (C_q), 126.3, 125.6 (CH_{ar}); 123.3 (CH), 120.5 (C_5), 112.8 (CH_{ar}), 89.0 ($C_{1'}$), 82.2, 80.5 (C_q); 80.3 ($C_{4'}$), 80.1 ($C_{2'}$), 78.4 ($C_{3'}$), 68.7, 68.6, 50.6 (CH_2); 48.4 ($C_{5'}$), 38.6, 37.3, 28.5, 28.3, 28.2 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{48}H_{63}N_{10}O_9$ $[M + H]^+$: 923.4779; Found: 923.4774.

1-[5′-Deoxy-2′,3′-di-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-*tert*-butoxycarbonyl)amino) phenyl) buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (28)

From alkyne **13** (198 mg, 0.510 mmol) and azido adenosine **22** (265 mg, 0.510 mmol), compound **28** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.4) as a yellow solid (397 mg, 86%). R_f = 0.33 (CH_2Cl_2/EtOH: 10/0.4); mp: 123–125 °C; $[\alpha]_D$ +8.1 (*c* 1.0, $CHCl_3$); IR (ATR): 2975, 1737, 1726, 1603, 1514, 1368, 1234, 1154, 1049, 985, 848, 800 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.19 (s, 1H, H_2), 7.72 (s, 1H, H_8), 7.34–7.29 (m, $4H_{ar}$), 7.21 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.20 (s, 1H, CH), 6.82 (dd, 1H, J = 10.4, 15.2 Hz, CH═), 6.71 (dd, 1H, J = 10.4, 15.2 Hz, CH═), 6.62 (br s, 1H, NH), 6.56 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 6.50 (d, 2H, J = 15.2 Hz, 2CH═), 6.10 (d, 1H, J = 2.8 Hz, $H_{1'}$), 5.89 (s, 2H, NH_2), 4.74–4.73 (m, 2H, CH_2), 4.70 (dd, 1H, J = 2.8, 4.0 Hz, $H_{2'}$), 4.55–4.42 (m, 4H, $H_{3'}$ + $H_{4'}$ + $2H_{5'}$), 4.32 (d, 1H, J = 16.8 Hz, CHCO), 4.24 (d, 1H, J = 16.8 Hz, CHCO), 4.21 (d, 1H, J = 16.8 Hz, CHCO), 4.19 (d, 1H, J = 16.8 Hz, CHCO), 2.89 (s, 3H, CH_3), 1.51 (s, 9H, *t*Bu), 1.48 (s, 9H, *t*Bu), 1.38 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 169.6, 169.5, 155.6 (C_q); 153.0 (C_2), 152.8, 149.3, 148.4, 145.2 (C_q); 140.4 (C_8), 137.4, 132.9 (C_q); 132.5, 130.1, 128.8 (CH═); 127.6, 126.9 (CH_{ar}); 126.6 (C_q), 125.8 (CH═), 123.3 (CH), 120.5 (C_5), 118.7, 112.8 (CH_{ar}); 89.0 ($C_{1'}$), 82.2, 80.7 (C_q); 80.3 ($C_{4'}$), 80.1 ($C_{2'}$), 78.4 ($C_{3'}$), 68.7, 68.6, 50.6 (CH_2); 48.4 ($C_{5'}$), 38.6, 28.5, 28.3, 28.2 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{47}H_{61}N_{10}O_9$ $[M + H]^+$: 909.4623; Found: 909.4618.

1-[5′-Deoxy-3′-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl-*N*-*tert*-butoxycarbonyl)amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (29)

From alkyne **14** (44 mg, 0.109 mmol) and azido adenosine **24** (44 mg, 0.109 mmol), compound **29** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.6) as a yellow solid (73 mg, 83%). R_f = 0.31 (CH_2Cl_2/EtOH: 10/0.5); mp: 139–141 °C; $[\alpha]_D$ −7.2 (*c* 1.0, $CHCl_3$); IR (ATR): 2972, 2921, 1692, 1640, 1602, 1511, 1367, 1249, 1146, 1107, 985, 846 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.18 (s, 1H, H_2), 7.67 (s, 1H, H_8), 7.33 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.21 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.16 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.07 (s, 1H, CH═), 6.87 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.71 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.61–6.49 (m, 4H, 2CH═, $2H_{ar}$), 6.13 (s, 2H, NH_2), 5.89 (d, 1H, *J* = 2.0 Hz, $H_{1'}$), 4.73 (dd, 1H, *J* = 4.4, 14.8 Hz), 4.62–4.58 (m, 2H), 4.52–4.47 (m, 2H), 4.43–4.34 (m, 2H), 4.36 (d, 1H, *J* = 17.2 Hz, CHCO), 4.11 (d, 1H, *J* = 17.2 Hz, CHCO), 3.64 (s, 1H, OH), 3.24 (s, 3H, CH_3), 2.85 (s, 3H, CH_3), 1.46 (s, 9H, *t*Bu), 1.45 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 171.4, 155.7, 154.8 (C_q); 153.1 (C_2), 149.2, 148.4, 145.2, 142.7 (C_q); 140.2 (C_8), 134.9 (C_q), 133.0, 129.9, 129.8 (CH═); 127.6 (CH_{ar}), 126.4 (C_q), 126.3 (CH_{ar}), 125.6 (CH═), 125.5 (CH_{ar}), 123.3 (CH), 120.3 (C_5), 112.7 (CH_{ar}), 90.3 ($C_{1'}$), 83.4 (C_q), 81.5 ($C_{4'}$), 80.5 (C_q), 79.8 ($C_{3'}$), 72.9 ($C_{2'}$), 69.9, 50.3 (CH_2); 48.3 ($C_{5'}$), 38.5, 37.3, 28.4, 28.1 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{42}H_{52}N_{10}O_7$ $[M + H]^+$: 809.4098; Found: 809.4093.

1-[5′-Deoxy-3′-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-*tert*-butoxycarbonyl)amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (30)

From alkyne **13** (41 mg, 0.106 mmol) and azido adenosine **23** (43 mg, 0.106 mmol), compound **30** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.6) as a yellow solid (71 mg, 84%). R_f = 0.31 (CH_2Cl_2/EtOH: 10/0.5); mp: 123–125 °C; $[\alpha]_D$ −5.8 (*c* 1.0, $CHCl_3$); IR (ATR): 3680 (br), 3303 (br), 2987, 2900, 1726, 1605, 1514, 1369, 1241, 1159, 1050 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.17 (s, 1H, H_2), 7.67 (s, 1H, H_8), 7.29 (m, 4H, H_{ar}), 7.19 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.07 (s, 1H, CH═), 6.79 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.69 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.51 (d, 2H, *J* = 9.2 Hz, $2H_{ar}$), 6.48 (d, 1H, *J* = 15.2 Hz, CH═), 6.46 (d, 1H, *J* = 15.2 Hz, CH═), 6.30 (br s, 2H, NH_2), 5.89 (d, 1H, *J* = 2.4 Hz, $H_{1'}$), 4.72 (dd, 1H, *J* = 4.4, 14.6 Hz), 4.63 (dd, 1H, *J* = 2.4, 4.8 Hz, $H_{2'}$), 4.59 (dd, 1H, *J* = 3.2, 14.4 Hz), 4.51–4.33 (m, 4H), 4.35 (d, 1H, *J* = 17.2 Hz, CHCO), 4.11 (d, 1H, *J* = 17.2 Hz, CHCO), 3.63 (br s, 1H, OH), 2.83 (s, 3H, CH_3), 1.50 (s, 9H, *t*Bu), 1.45 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 171.3, 155.8 (C_q); 153.0 (C_2), 152.8, 149.1, 148.3, 145.1 (C_q); 140.2 (C_8), 137.4, 132.8 (C_q); 132.4, 130.1, 128.7 (CH═); 127.6, 126.8 (CH_{ar}); 126.5 (C_q), 125.7 (CH═), 123.3 (CH), 120.2 (C_5), 118.7, 112.7 (CH_{ar}); 90.2 ($C_{1'}$), 83.3 (C_q), 81.5 ($C_{4'}$), 80.6 (C_q), 79.8 ($C_{3'}$), 72.8 ($C_{2'}$), 69.9, 50.3 (CH_2); 48.2 ($C_{5'}$), 38.4, 28.4, 28.1 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{41}H_{51}N_{10}O_7$ $[M + H]^+$: 795.3942; Found: 795.3937.

1-[5′-Deoxy-2′-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl-*N*-*tert*-butoxycarbonyl)amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (31)

From alkyne **14** (64 mg, 0.159 mmol) and azido adenosine **23** (65 mg, 0.159 mmol), compound **31** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.6) as a yellow solid (112 mg, 87%). R_f = 0.12 (CH_2Cl_2/EtOH: 10/0.6); mp: 109–111 °C; $[\alpha]_D$ +15.1 (*c* 1.0, $CHCl_3$); IR (ATR): 3321, 3171, 2974, 2930, 1734, 1691, 1641, 1602, 1511, 1367, 1247, 1145, 985, 910, 843 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.27 (s, 1H, H_2), 7.70 (s, 1H, H_8), 7.35 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.26 (s, 1H, CH), 7.23 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.17 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 6.87 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.73 (dd, 1H, *J* = 10.4, 15.2 Hz, CH═), 6.63 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 6.54 (d, 1H, *J* = 15.2 Hz, CH═), 6.53 (d, 1H, *J* = 15.2 Hz, CH═), 6.02 (d, 1H, *J* = 4.8 Hz, $H_{1'}$), 5.87 (s, 2H, NH_2), 4.77 (dd, 1H, *J* = 4.2, 14.6 Hz), 4.72 (dd, 1H, *J* = 6.2, 14.6 Hz), 4.61 (t, 1H, *J* = 4.8 Hz), 4.57 (br s, 2H), 4.45–4.37 (m, 2H), 4.24 (d, 1H, *J* = 17.2 Hz, CHCO), 3.99 (d, 1H, *J* = 17.2 Hz, CHCO), 3.25 (s, 3H, CH_3), 2.95 (s, 3H, CH_3), 1.45 (s, 18H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 170.5, 155.9, 154.7 (C_q); 153.1 (C_2), 149.3, 148.4, 145.1, 142.6 (C_q); 140.0 (C_8), 134.9 (C_q), 132.9, 129.8 (CH═); 127.6 (CH_{ar}), 126.3 (C_q), 126.2, 125.4 (CH_{ar}); 123.3 (CH), 120.3 (C_5), 112.8 (CH_{ar}), 88.1 ($C_{1'}$), 83.2 (C_q), 83.0 ($C_{4'}$), 82.2 ($C_{2'}$), 80.4 (C_q), 70.4 ($C_{3'}$), 69.5, 51.1 (CH_2); 48.2 ($C_{5'}$), 38.5, 37.2, 28.4, 28.0 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{42}H_{53}N_{10}O_7$ $[M + H]^+$: 809.4098; Found: 809.4093.

1-[5′-Deoxy-2′-*O*-(*tert*-butoxycarbonylmethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-*tert*-butoxycarbonyl)amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (32)

From alkyne **13** (56 mg, 0.144 mmol) and azido adenosine **23** (59 mg, 0.144 mmol), compound **32** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 10/0.6) as a yellow solid (100 mg, 87%). R_f = 0.12 (CH_2Cl_2/EtOH: 10/0.6); mp: 132–134 °C; $[\alpha]_D$ +13.5 (*c* 1.0, $CHCl_3$); IR (ATR): 1725, 1632, 1601, 1514, 1368, 1239, 1156, 1052, 985, 843 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz): δ 8.27 (s, 1H, H_2), 7.69 (s, 1H, H_8), 7.34–7.29 (m, $4H_{ar}$), 7.26 (s, 1H, CH═), 7.22 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 6.84 (dd, 1H, *J* = 10.6, 15.2 Hz, CH═), 6.72 (dd, 1H, *J* = 10.6, 15.2 Hz, CH═), 6.62 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 6.60 (br s, 1H, NH), 6.51 (d, 1H, *J* = 15.2 Hz, CH═), 6.50 (d, 1H, *J* = 14.8 Hz, CH═), 6.02 (d, 1H, *J* = 5.2 Hz, $H_{1'}$), 5.92 (br s, 2H, NH_2), 4.77 (dd, 1H, *J* = 4.4, 14.6 Hz), 4.71 (dd, 1H, *J* = 6.4, 14.6 Hz), 4.60 (t, 1H, *J* = 4.8 Hz), 4.57 (br s, 2H), 4.54–4.53 (m, 1H), 4.44–4.41 (m, 1H), 4.39–4.37 (m, 1H), 4.23 (d, 1H, *J* = 17.2 Hz, CHCO), 3.99 (d, 1H, *J* = 17.2 Hz, CHCO), 2.94 (s, 3H, CH_3), 1.52 (s, 9H, *t*Bu), 1.44 (s, 9H, *t*Bu); RMN ^{13}C ($CDCl_3$, 100 MHz): δ 170.6, 155.9 (C_q); 153.1 (C_2), 152.8, 149.4, 148.3, 145.2 (C_q); 140.1 (C_8), 137.4, 132.8 (C_q); 132.4, 130.1, 128.7 (CH═); 127.5, 126.8 (CH_{ar}); 126.5 (C_q), 125.7 (CH═), 123.3 (CH), 120.4 (C_5), 118.7, 112.9 (CH_{ar}); 88.1 ($C_{1'}$), 83.3 (C_q), 83.1 ($C_{4'}$), 82.3 ($C_{2'}$), 80.6 (C_q), 70.4 ($C_{3'}$), 69.6,

51.1 (CH_2); 48.3 ($C_{5'}$), 38.6, 28.4, 28.0 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{41}H_{51}N_{10}O_7$ $[M + H]^+$: 795.3942; Found: 795.3937.

1-[5′-Deoxy-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-*tert*-butoxycarbonyl)amino)phenyl)buta-1,3-dienyl)phenyl)) aminomethyl]-1*H*-1,2,3-triazole (33)

From alkyne **13** (57 mg, 0.15 mmol) and azido adenosine **21** (43 mg, 0.15 mmol), compound **33** was obtained after purification by column chromatography over silica gel (CH_2Cl_2/EtOH: 9/1) as a yellow solid (80 mg, 80%). R_f = 0.49 (CH_2Cl_2/EtOH: 9/1); mp: 162–164 °C; IR (ATR): 3347 (br s), 1709, 1641, 1603, 1514, 1369, 1314, 1237, 1159, 1053, 980 cm^{-1}; RMN ^1H (DMSO-d_6, 400 MHz): δ 9.40 (s, 1H, NH), 8.21 (s, 1H, H_2), 8.14 (s, 1H, H_8), 7.75 (s, 1H, CH), 7.42–7.34 (m, 6H, NH_2 + $4H_{ar}$), 7.23 (d, 2H, *J* = 8.7 Hz, $2H_{ar}$), 6.89 (dd, 1H, *J* = 10.6, 15.6 Hz, CH═), 6.77 (dd, 1H, *J* = 10.5, 15.1 Hz, CH═), 6.69 (d, 2H, *J* = 9.2 Hz, $2H_{ar}$), 6.53 (d, 1H, *J* = 15.1 Hz, CH═), 6.52 (d, 1H, *J* = 15.1 Hz, CH═), 5.88 (d, 1H, *J* = 5.0 Hz, $H_{1'}$), 5.61 (d, 1H, *J* = 6.0 Hz, $H_{2'}$), 5.47 (d, 1H, *J* = 4.6 Hz, $H_{3'}$), 4.71–4.66 (m, 2H, CH_2), 4.65–4.62 (m, 1H, $H_{4'}$), 4.51 (s, 2H, 2OH), 4.23–4.20 (m, 2H, $2H_{5'}$), 2.91 (s, 3H, CH_3), 1.47 (s, 9H, *t*Bu); RMN ^{13}C (DMSO-d_6, 100 MHz): δ 156.1 (C_q), 152.7 (C_2), 149.3, 148.3, 143.7 (C_q); 139.9 (C_8), 138.5, 132.2 (C_q); 131.5, 129.8, 128.3 (CH═); 127.3, 126.4 (CH_{ar}); 125.4, 125.2 (C_q); 123.6 (CH), 119.2 (C_5), 118.2, 112.6 (CH_{ar}); 87.8 ($C_{1'}$), 82.2 ($C_{2'}$), 79.1 (C_q), 72.6 ($C_{4'}$) 70.9 ($C_{3'}$), 51.2 (CH_2), 46.8 ($C_{5'}$), 38.1, 28.1 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{35}H_{40}N_{10}O_5$ $[M + H]^+$: 681.3261; Found: 681.3163.

1-[5′-Deoxy-2′,3′-di-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl)amino)phenyl)buta-1,3-dienyl) phenyl))aminomethyl]-1*H*-1,2,3-triazole (1)

Deprotection of compound **27** (118 mg, 0.128 mmol) according to the general procedure afforded **1** (91 mg, 100%) as a red solid. mp: 171–173 °C; IR (ATR): 2915 (br), 1737, 1688, 1597, 1509, 1462, 1420, 1230, 1151, 994 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.40 (s, 1H, H_2), 8.39 (s, 1H, H_8), 8.02 (s, 1H, CH), 7.68 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.54 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.51 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.42 (d, 2H, *J* = 8.8 Hz, $2CH_{ar}$), 7.13–7.02 (m, 2H, 2CH═), 6.79 (d, 1H, *J* = 14.8 Hz, CH═), 6.72 (d, 1H, *J* = 14.4 Hz, CH═), 6.23 (d, 1H, *J* = 4.4 Hz, $H_{1'}$), 4.90–4.81 (m, 5H, CH_2 + $H_{2'}$ + $2H_{5'}$), 4.50–4.35 (m, 6H, $H_{3'}$ + $H_{4'}$ + $2CH_2$), 3.32 (s, 3H, CH_3), 3.09 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 172.5, 172.3, 151.7, 149.6 (C_q); 145.3 (C_2), 144.5 (C_8), 141.2, 140.3, 139.4, 139.0, 137.1 (C_q); 133.2, 133.0, 132.3, 132.0 (CH═); 129.3, 129.0, 123.6, 122.4 (CH_{ar}); 121.0 (C_5), 89.8 ($C_{1'}$), 82.6 ($C_{4'}$), 81.6 ($C_{2'}$), 79.1 ($C_{3'}$), 68.4, 54.4 (CH_2); 52.6 ($C_{5'}$), 44.3, 37.8 (CH_3); HRMS (ESI) *m*/*z*: Calcd for C35H38$N_{10}O_7$ $[M + H]^+$: 711.3003; Found: 711.2998.

1-[5′-Deoxy-2′,3′-di-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-amino)phenyl)buta-1,3-dienyl)phenyl)) aminomethyl]-1*H*-1,2,3-triazole (2)

Deprotection of compound **28** (102 mg, 0.112 mmol) according to the general procedure afforded **2** (99 mg, 100%) as a red solid. mp: 171–173 °C; IR (ATR): 2923 (br), 2851, 1741, 1688, 1605, 1509, 1426, 1232, 1153, 1086, 993, 850 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.35(s, 1H, H_2), 8.33 (s, 1H, H_8), 7.94 (s, 1H, CH═), 7.65 (d, 2H, *J* = 8.8 Hz, $2CH_{ar}$), 7.52 (d, 2H, *J* = 8.8 Hz, $2CH_{ar}$), 7.45 (d, 2H, *J* = 8.8 Hz, $2CH_{ar}$), 7.35 (d, 2H, *J* = 8.8 Hz, $2CH_{ar}$), 7.11–7.00 (m, 2H, 2CH═), 6.75 (d, 1H, *J* = 14.8 Hz, CH═), 6.70 (d, 1H, *J* = 14.8 Hz, CH═), 6.20 (d, 1H, *J* = 4.4 Hz, $H_{1'}$), 4.81–4.76 (m, 5H), 4.48–4.31 (m, 6H), 3.05 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 172.4, 172.1, 151.6, 149.7 (C_q); 145.4 (C_2), 145.2 (C_q), 144.5 (C_8), 140.5, 140.2, 139.5 (C_q); 133.5, 132.7, 132.6, 131.7 (CH═); 130.8 (C_q), 129.8 (CH═), 129.0, 124.5, 123.2 (CH_{ar}); 121.2 (C_5), 89.7 ($C_{1'}$), 82.5 ($C_{4'}$), 81.6 ($C_{2'}$), 79.2 ($C_{3'}$), 68.6, 68.5, 55.1 (CH_2); 52.9 ($C_{5'}$), 45.1 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{34}H_{37}N_{10}O_7$ $[M + H]^+$: 697.2846; Found: 697.2841.

1-[5′-Deoxy-3′-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl)amino)phenyl)buta-1,3-dienyl)phenyl)) aminomethyl]-1*H*-1,2,3-triazole (3)

Deprotection of compound **29** (35 mg, 0.043 mmol) according to the general procedure afforded **3** (30 mg, 100%) as a red solid. mp: 172–174 °C; IR (ATR): 3327 (br), 2925, 1737, 1687, 1604, 1509, 1460, 1427, 1242, 1142, 1085, 1000, 824 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.43 (s, 1H, H_2), 8.36 (s, 1H, H_8), 8.03 (s, 1H, CH═), 7.70 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.59 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.50 (d, 2H, *J* = 8.8 Hz, $2H_{ar}$), 7.48 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.16–7.09 (m, 2H, 2CH═), 6.85–6.72 (m, 2H, 2CH═), 6.05 (d, 1H, *J* = 5.2 Hz, $H_{1'}$), 4.89–4.80 (m, 4H), 4.73 (t, 1H, *J* = 5.0 Hz), 4.53–4.49 (m, 1H), 4.42 (d, 1H, *J* = 17.0 Hz, CHCO), 4.35 (d, 1H, *J* = 17.0 Hz, CHCO), 4.28 (t, 1H, *J* = 5.0 Hz), 3.35 (s, 3H, CH_3), 3.09 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 173.0, 151.6, 149.7 (C_q); 145.4 (C_2), 144.3 (C_8), 140.6, 140.3, 138.2, 137.1 (C_q); 133.4, 132.9, 132.6, 132.2 (CH═); 131.7 (C_q), 129.4 (CH), 129.2, 129.1, 123.7, 123.3 (CH_{ar}); 120.9 (C_5), 90.8 ($C_{1'}$), 82.6 ($C_{4'}$), 80.9 ($C_{3'}$), 74.3 ($C_{2'}$), 68.9, 55.0 (CH_2); 52.9 ($C_{5'}$), 45.0, 37.9 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{33}H_{36}N_{10}O_5$ $[M + H]^+$: 653.2948; Found: 653.2943.

1-[5′-Deoxy-3′-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-amino)phenyl)buta-1,3-dienyl)phenyl)) aminomethyl]-1*H*-1,2,3-triazole (4)

Deprotection of compound **30** (31 mg, 0.039 mmol) according to the general procedure afforded **4** (25 mg, 100%) as a red solid. mp: 182–184 °C; IR (ATR): 3320 (br), 3080 (br), 2930, 2586, 1738, 1688, 1601, 1510, 1427, 1239, 1143, 1084, 993, 860 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.43 (s, 1H, H_2), 8.38 (s, 1H, H_8), 8.09 (s, 1H, CH═), 7.65 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.57–7.52 (m, $4H_{ar}$), 7.42 (d, 2H, *J* = 8.4 Hz, $2H_{ar}$), 7.10–7.04 (m, 2H, 2CH═), 6.78 (d, 1H, *J* = 14.0 Hz, CH═), 6.70 (d, 1H, *J* = 14.0 Hz, CH═), 6.04 (d, 1H, *J* = 4.4 Hz, $H_{1'}$), 4.95–4.80 (m, 4H), 4.74–4.73 (m, 1H), 4.51–4.49 (m, 1H), 4.43 (d, 1H, *J* = 17.0 Hz, CHCO), 4.36 (d, 1H, *J* = 17.0 Hz, CHCO), 4.30–4.29 (m, 1H), 3.35 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 172.9, 151.6, 149.7 (C_q); 145.4 (C_2), 144.3 (C_8), 140.7, 140.2, 139.6, 138.2 (C_q); 133.6, 132.7, 131.8 (CH═); 130.9 (C_q), 129.4 (CH_{ar}), 129.1, 124.6, 123.3 (CH_{ar}); 120.9 (C_5), 90.8 ($C_{1'}$), 82.6 ($C_{4'}$), 80.9 ($C_{3'}$), 74.3 ($C_{2'}$), 68.9, 55.0 (CH_2); 52.9 ($C_{5'}$), 45.1 (CH_3); HRMS

(ESI) *m*/*z*: Calcd for $C_{32}H_{35}N_{10}O_5$ $[M + H]^+$: 639.2792; Found: 639.2786.

1-[5′-Deoxy-2′-di-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-(*N*-methyl)amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (5)

Deprotection of compound **31** (42 mg, 0.052 mmol) according to the general procedure afforded **5** (34 mg, 100%) as a red solid. mp: 116–118 °C; IR (ATR): 3060 (br), 2924, 1736, 1689, 1680, 1600, 1510, 1423, 1241, 1141, 857 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.42 (s, 1H, H_2), 8.41 (s, 1H, H_8), 7.98 (d, 1H, J = 2.4 Hz, CH), 7.70 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.57 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.50 (d, 2H, J = 8.4 Hz, $2H_{ar}$), 7.45–7.43 (m, $2H_{ar}$), 7.16–7.06 (m, 2H, 2CH═), 6.85–6.71 (m, 2H, 2CH═), 6.23 (d, 1H, J = 4.4 Hz, $H_{1'}$), 4.83–4.80 (m, 4H), 4.67 (t, 1H, J = 4.6 Hz), 4.45 (t, 1H, J = 5.2 Hz), 4.38 (d, 1H, J = 17.0 Hz, CHCO), 4.36–4.30 (m, 1H), 4.30 (d, 1H, J = 17.0 Hz, CHCO), 3.35 (s, 3H, CH_3), 3.09 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 172.6, 151.6, 149.7 (C_q); 145.4 (C_2), 144.7 (C_8), 140.6, 140.3, 140.2, 138.4, 137.0 (C_q); 133.4, 133.0, 132.6, 132.2 (CH═); 129.5 (CH═), 129.3, 129.1, 123.7, 123.3 (CH_{ar}); 121.1 (C_5), 89.7 ($C_{1'}$), 84.1 ($C_{4'}$), 83.3 ($C_{2'}$), 71.4 ($C_{3'}$), 69.0, 55.1 (CH_2); 52.9 ($C_{5'}$), 45.0, 37.9 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{33}H_{37}N_{10}O_5$ $[M + H]^+$: 653.2952; Found: 653.2943.

1-[5′-Deoxy-2′-*O*-(carboxymethyl)-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (6)

Deprotection of compound **32** (49 mg, 0.062 mmol) according to the general procedure afforded **6** (40 mg, 100%) as a red solid, mp: 181–183 °C; IR (ATR): 3345 (br), 1736, 1688, 1610, 1509, 1457, 1423, 1232, 1141, 995, 848, 732 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.41 (s, 1H, H_2), 8.39 (s, 1H, H_8), 7.99 (s, 1H, CH), 7.66 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.65 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.42 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.40 (d, 2H, J = 8.8 Hz, $2H_{ar}$), 7.12–7.10 (m, 2H, 2CH═), 6.84–6.69 (m, 2H, 2CH═), 6.21 (d, 1H, J = 4.4 Hz, $H_{1'}$), 4.90–4.80 (m, 4H), 4.68 (t, 1H, J = 4.8 Hz), 4.43 (t, 1H, J = 5.2 Hz), 4.37–4.26 (m, 3H), 3.32 (s, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 173.9, 151.7, 149.7 (C_q); 145.3 (C_2), 144.6 (C_8), 141.0, 139.8, 138.8 (C_q); 133.3, 132.9, 132.2, 131.9 (CH═); 130.9 (C_q), 129.1, 129.0 (CH_{ar}); 128.9 (CH═), 124.6, 122.5 (CH_{ar}); 121.1 (C_5), 89.8 ($C_{1'}$), 84.2 ($C_{4'}$), 83.3 ($C_{2'}$), 71.4 ($C_{3'}$), 68.9, 54.5, 52.8 (CH_2); 44.4 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{32}H_{35}N_{10}O_5$ $[M + H]^+$: 639.2792; Found: 639.2786.

1-[5′-Deoxy-adenosin-5′-yl]-4-[(*N*-methyl-*N*-((4-(4-(4-amino)phenyl)buta-1,3-dienyl)phenyl))aminomethyl]-1*H*-1,2,3-triazole (7)

Deprotection of compound **33** (38 mg, 0.056 mmol) according to the general procedure afforded **7** (34 mg, 100%) as a red solid; mp: 174–176 °C; IR (ATR): 2957 (br s), 2597, 1686, 1600, 1506, 1413, 1225, 1130, 1064 cm^{-1}; RMN ^1H (CD_3OD, 400 MHz): δ 8.39 (s, 1H, H_2), 8.35 (s, 1H, H_8), 7.98 (s, 1H, CH═), 7.64 (d, 2H, J = 8.7 Hz, $2CH_{ar}$), 7.55 (d, 2H, J = 8.7 Hz, $2CH_{ar}$), 7.43 (d, 2H, J = 9.2 Hz, $2CH_{ar}$), 7.37 (d, 2H, J = 8.7 Hz, $2CH_{ar}$), 7.08–7.05 (m, 2H, 2CH═), 6.79–6.68 (m, 2H, 2CH═), 6.00 (d, 1H, J = 4.6 Hz, $H_{1'}$), 4.85–4.79 (m, 2H, $H_{5'}$ + $H_{4'}$), 4.64 (t, 1H, J = 4,6 Hz, $H_{2'}$), 4.32–4.29 (dd, 2H, J = 5.0 Hz, 8.7 Hz, $H_{3'}$ + $H_{5'}$), 3.61 (s, 2H, CH_2), 3.29–3.28 (m, 3H, CH_3); RMN ^{13}C (CD_3OD, 100 MHz): δ 151.8, 149.7 (C_q); 145.4 (C_2), 144.5 (C_8), 140.7, 140.2, 139.7, 138.5 (C_q); 133.6, 132.9, 132.5, 131.9 (CH═); 130.9 (C_q), 129.1, 129.0, 124.6 (CH_{ar}); 124.5 (CH═), 122.8 (CH_{ar}), 121.0 (C_5), 91.2 ($C_{1'}$), 84.3 ($C_{2'}$), 75.0 ($C_{4'}$), 72.5 ($C_{3'}$), 54.7 (CH_2), 53.0 ($C_{5'}$), 44.7 (CH_3); HRMS (ESI) *m*/*z*: Calcd for $C_{30}H_{32}N_{10}O_3$ $[M + H]^+$: 581.2737; Found: 581.2735.

Spectroscopic characterization of NT compounds

Absorption spectra of NTs in DMSO or Tris buffer (20 mM Tris-HCl, pH 7.4) were carried out at 22 °C with a Uvikon spectrophotometer. Fluorescence excitation and emission spectra under one-photon excitation condition (1-PE) were recorded at 22 °C on an Eclipse (Varian) fluorimeter. Fluorescence quantum yields (ϕ) were measured using Coumarin-1 (Sigma) in ethanol as a reference ($\lambda_{max,exc}$ = 373 nm; λ_{em} = 447 nm). Two-photon excitation (2-PE) and emission spectra were recorded using a home-built set-up.[10,21] Briefly, a 80 MHz mode-locked Mai-Tai® Ti:Sapphire tunable laser (690–1040 nm, 100 fs laser pulse; Spectra Physics) was focused onto the sample (80 μL) placed in a quartz micro cell. The two-photon fluorescence signal was collected at 90°, filtered by a Semrock FF01-842/SP filter – to reject the residual excitation light – and focused into an optical fiber connected to a QE65000 spectrometer (Ocean Optics). Two-photon absorption cross-sections (σ^2) of NTs were determined as previously described.[10]

Binding affinity evaluation

The apparent binding constants of the NTs to the neuronal (nNOS) and endothelial (eNOS) isoforms of nitric oxide synthase (NOS) were assessed by changes in NT intrinsic fluorescence. NOS proteins were recombinantly expressed and purified as described.[22] NTs were dissolved in DMSO to make ~10 mM stock solutions, which were diluted to 300 μM in buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl). Aliquots (1 μL) were added to 100 μL buffer containing NOS protein (5 μM), and fluorescence emission spectra were recorded from 430–700 nm (excitation = 420 nm; slit widths = 5 nm) on a Shimadzu RF-5301PC fluorometer. Spectra of intrinsic protein fluorescence and unbound NT were subtracted from protein/NT spectra and the fluorescence at 460 nm was plotted against NT concentration. Resultant curves were fit to a rectangular hyperbola $y = m1 \times x/(x + m2)$, where $m1$ is maximum fluorescence and $m2$ is the apparent binding constant, and apparent binding constant was determined.

Cell culture and fluorescence imaging of NTs in HUVEC and HeLa cells

Endothelial cells (HUVEC; Sigma) were cultured in Petri dishes coated with 0.2% gelatin in Endothelial Cell Growth Medium (Cell Application).[23] HeLa cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco®) sup-

plemented with 10% Fetal Bovin Serum (FBS; Gibco®) and 1% penicillin–streptomycin (Gibco®). HUVEC and HeLa cells were cultured at 37 °C (5% CO_2) to ≈95% confluence. In all experiments with HUVECs, the cells were used within passage five. Two-photon fluorescence imaging of NT compounds in living HUVEC and HeLa cells was performed using a multiphoton imaging set-up previously described.[10,19] Briefly, HUVEC and HeLa cells were plated in glass bottom dishes (WillCo-dish; WillCo Wells) and treated for varying times with NT compounds at a final concentration of 5 μM. Two-photon fluorescence images were obtained using a SP2 confocal microscope (Leica MicroSystems) equipped with an oil immersion ×63 objective (numerical aperture, 1.32) and an incubation chamber (37 °C, CO_2 5%). The excitation source was a 80 MHz mode-locked Mai-Tai® Ti:Sapphire tunable laser (720–920 nm, 100 fs laser pulse; Spectra Physics) tuned to 760 nm (emission slit setting: 450—550 nm).

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