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Customizable and regioselective one-pot *N–H* functionalization of DNA nucleobases to create a library of nucleobase derivatives for biomedical applications

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Abstract: DNA is one of the most exciting biomolecules in nature for developing supramolecular biofunctional nanoarchitectures owing to the highly specific and selective interactions between complementary Watson-Crick base pairing. Herein, simple and one-pot synthetic procedures have been implemented for producing a library of DNA nucleobase derivatives endowed with reactive functional groups for bioconjugation and cross-linking strategies with other (bio)molecules. Purine and pyrimidine molecules have been regioselectively *N–H* functionalized either via *N*-alkylation, *N*-allylation, *N*-propargylation or Michael-type reactions and structurally characterized. The influence of the reaction conditions was assessed and discussed. The *in vitro* biocompatibility of the native and nucleobase derivatives was evaluated by culturing them with human fibroblasts, revealing their cytocompatibility. The library of nucleobase derivatives holds great promise for being coupled to different biomolecules, including biopolymeric materials, lipids, and peptides, thus potentially leading to modular supramolecular nanobiomaterials for biomedicine.

Introduction

Nature provides us with a unique source of biomolecules that are inherently responsible for supporting and orchestrating the biological processes that govern life on Earth. Among them, DNA is unequivocally one of the most powerful biomolecules that has extended well beyond its classical key role on storing, encoding, and conveying the genetic information in biological systems through its basic molecular building blocks – the natural nucleobases [adenine (**A**), guanine (**G**), thymine (**T**), and cytosine (**C**) (Figure 1)].[1,2]

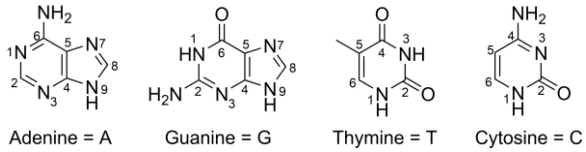


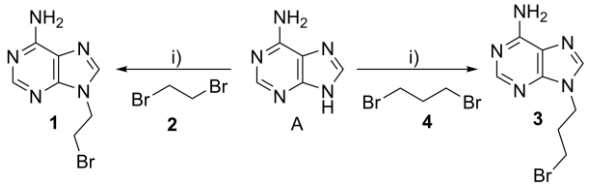
Figure 1. Purine (A and G) and pyrimidine (T and C) of DNA nucleobases subjected to *N–H* chemical functionalization.

Over the last two decades, the interest in the rational design and development of DNA-based supramolecular multifunctional nanoarchitectures has notably increased due to recognition of the DNA as one of the smartest, versatile and powerful natural building blocks in the biomedical arena.[3-7] However, challenges associated to the nucleobases themselves, including the preservation of hydrogen bonding capabilities, the regioselective introduction of functional groups in high yield, and the poor solubility in aqueous media and commonly used organic solvents, remain as the major bottlenecks for modulating the nucleobases' reactivity, physicochemical and biological properties.[8-13] As such, increasing efforts have been devoted to improve the solubility of the nucleobases. For instance, it has been reported that the addition of appropriate solubilizing groups such as 2-ethylhexyl chains ensures good solubility to the nucleobases in organic solvents.[10] The nitrogen atoms in purine and pyrimidine rings reveal varying degrees of nucleophilicity and, thus, react differently with several electrophilic compounds, such as alkylating or acylating agents.[9] The *N*-alkylation of nucleobases has been receiving considerable attention since this reaction is a rapid and direct route to access the acyclic nucleosides frequently used as chemotherapeutic agents.[9,14-16] To achieve the *N*-alkylation of the nucleobases, a wide variety of carbon-electrophiles have been used, including alkyl and aryl halides,[9,17] α,β-unsaturated esters,[18] carbonates,[19] and carboxamide.[20]

However, most of them are known to be toxic and carcinogenic, which extensively limits their use in the biomedical arena. Recently, it has been shown that primary alcohols are appropriate candidates to react with nucleobases, leading to N-alkyl derivatives in good yield and with low toxicity.[21]

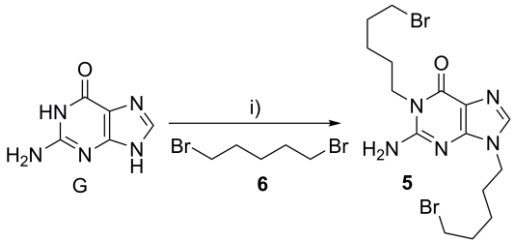
The chemical modification of nucleobases with either terminal alkyne,[22-24] acrylate,[25-27] allyl,[28,29] acrylonitrile[26,30-32] and thiol[33] functional groups at the secondary amine has been explored in the last decades, and in most of the cases, **A** and **T** have been at the limelight. From the chemical functionalization standpoint, the major bottlenecks associated to these functionalization strategies include the development of efficient synthetic methodologies for the successful introduction of reactive terminals at **C** and **G**, as well as simple and direct methods for the regioselective functionalization of the DNA nucleobases, aiming to preserve their hydrogen bonding capabilities. As such, there is still plenty of room for improving the known and developing new and innovative synthetic approaches for enabling the functionalization of all DNA nucleobases. The conjugation of the functionalized nucleobases with either synthetic or natural polymers, lipids and/or peptides enable the development of complex supramolecular biofunctional nanomaterials well-suited for a wide range of biomedical applications.[25,34-39]

Herein we report the one-pot synthesis of a new library of nucleobase derivatives by regioselective *N–H* functionalization of the DNA nucleobases with either alkyl, allyl, propargyl or propanenitrile functional groups via *N*-alkylation, *N*-allylation, *N*-propargylation or Michael-type reactions, respectively, aiming to extend the functionality of the nucleobases towards obtaining added-value biofunctional materials. Their chemical and structural characterization was performed by nuclear magnetic resonance (NMR), mass spectrometry (MS) and high-resolution mass spectrometry (HR-MS). Furthermore, the in vitro biocompatibility of the native and nucleobase derivatives was assessed using primary human dermal fibroblasts (HF), revealing their non-cytotoxicity and, thus suitability for addressing biomedical applications.



Scheme 1. Synthesis of *N*-alkylated **A** (**1**) and (**3**). i) K_2CO_3, dry DMF, r.t.. Yields: 53 % (**1**), 77 % (**3**).

Initial attempts to react **G** with 1,5-dibromopentane **6** followed a synthetic methodology like the one described by Kang *et al.*[27] using tetrabutylammonium iodide (TBAI) and C18-crown-6.[43] However, the reaction was not successful since only unmodified **G** was recovered (Table 1, entries 1-2). Therefore, some modifications were made. In particular, the reactions were carried out in two steps (Table 1, entries 3-4): firstly, **G** dissolution and deprotonation followed by the addition of compound **6**. Although in vestigial quantities, the *N*-alkylation reaction afforded the envisioned dialkylated guanine **5** (Scheme 2) solely when the compound **6** was added dropwise for 30 min at r.t. (Table 1, entry 4). The synthesis of the dialkylated product **5** (Scheme 2) in vestigial amounts could be explained by the deprotonation of the acidic N(3) and N(9) protons of **G**.



Scheme 2. Synthesis of *N*-dialkylated **G** (**5**). i) NaH (60% mineral oil), dry DMF, r.t., traces (**5**).

Results and Discussion

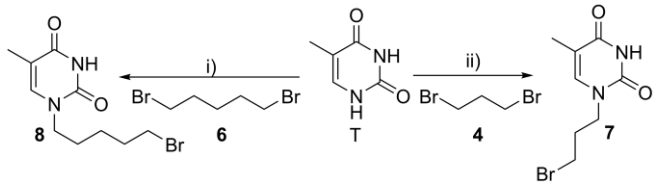
Functionalization of nucleobases via N-alkylation reaction

The synthesis of *N*-alkylated **A** followed previously reported synthetic procedures.[16,33,40] Briefly, **A** was treated with potassium carbonate (K_2CO_3) in anhydrous *N,N*-dimethylformamide (DMF), followed by the addition of 1,2-dibromoethane **2** at room temperature (r.t.) to obtain 9-(2-bromoethyl)-9*H*-purin-6-amine **1** in moderate yield (53%). **A** was also treated with 1,3-dibromopropane **4** to generate the 9-(3-bromopropyl)-9*H*-purin-6-amine **3** in good yield (77%) (Scheme 1), following a similar synthetic procedure.

The alkylation of **G** has revealed to be much more challenging. In fact, it has been scarcely investigated by the organic chemistry community due to its very low solubility in common organic solvents. Moreover, **G** oxidizes more readily than **A**. Its high melting point of 350 °C reflects the intermolecular hydrogen bonding between the oxo and amino groups in the crystal structure, being responsible for its insolubility in water.[41,42]

Some successful examples of **T** *N*-alkylations have been reported in the literature.[27,40,44] Following similar conditions to those reported in the literature, although using different alkylating agents, namely 1,2-dibromoethane **2** and 1,3-dibromopropane **4**, different reaction conditions were tested such as the use of phase transfer catalysts (PTC), solvents [water, dichloromethane, anhydrous DMF, dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF)], bases (K_2CO_3, NaOH and NaH), as well as distinct heating methods [conventional, microwave (MW) and ohmic heating]. However, in most cases no reaction occurred or the expected *N*-alkylated **T** was not obtained. Several alkylation conditions attempted without (Table 1, entry 5) or with phase-transfer agent (Table 1, entry 6) did not afford the desired alkylated **T**. The *N*-alkylation of **T** using K_2CO_3 in anhydrous DMSO, followed by the addition of 1,2-dibromopropane **4** at r.t., afforded **7** with 5% yield (Table 1, entry 7) (Scheme 3). We hypothesize that the addition of PTC such as TBAI, tetrabutylammonium bromide (TBAB), tetrabutylammonium acetate (TBAA), tetrabutylammonium hydroxide (TBAOH) and crown ether could offer a simple and efficient solution to counteract the low solubility of the nucleobases in common organic solvents and could facilitate the *N*-alkylation. The

attempts to couple 1,5-dibromopentane **6** to **T** at r.t. using the combinations K_2CO_3/TBAB and NaOH/TBAB were not successful. However, the increase in the reaction time, temperature and exchange of TBAB by TBAI led to 1-(5-bromopentyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione **8** in low yields (15-20%), as well as to the recovery of the starting material **T** (Table 1, entries 8-9).



Scheme 3. Synthesis of *N*-alkylated **T** (**7**) and (**8**). i) K_2CO_3, TBAI, dry DMF, r.t., ii) K_2CO_3, DMSO, r.t. Yields: 5 % (**7**), 20 % (**8**).

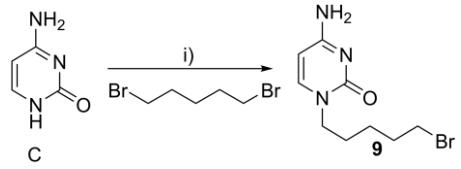
Table 1. Screening of the reaction conditions for the synthesis of *N*-alkylated nucleobases

Entry	Nucleobase	Catalyst (equiv)	Solvent	*T*	Base	Yield (%)
1[a]	G	TBAI (0.1)	MeOH	r.t., 65 h then reflux, 24 h	K_2CO_3	N.R.[b]
2	G	C18-crown-6	MeOH	reflux	K_2CO_3	N.R.[b]
3[a]	G	-	DMF	r.t., 72 h then 50 °C, 48 h	NaH[c]	N.R.[b]
4[a][d]	G	-	DMF	r.t., 72 h then 50 °C, 48 h	NaH[c]	Trace
5	T	-	DMF	r.t., 24 h then 80°C, 24 h	K_2CO_3	N.R.[b]
6	T	TBAB (0.06)	DMF	r.t., 25 h	K_2CO_3	N.R.[b]
7	T	-	DMSO	r.t., 24 h	K_2CO_3	5
8	T	TBAI (0.08)	DMF	40 °C, 48 h	K_2CO_3	15
9	T	TBAI (0.06)	DMF	r.t. 30 h	K_2CO_3	20
10	C	TBAA	DMF	r.t., overnight	-	N.R.[b]
11	C	TBAOH in 40% H_2O	DMF	r.t., 24 h	-	N.R.[b]
12	C	TBAOH in MeOH	DMF	r.t., 24 h	-	10
13	C	TBAI (0,08)	MeOH	r.t, 24 h	-	12
14[a]	C	TBAI (0.1)	MeOH	r.t., 65 h then 65 °C, 24 h	K_2CO_3	50

[a] Reaction was carried on in two steps. [b] No reaction. [c] NaH in 60% wt mineral oil. [d] Addition of 1,5-dibromopentane **6** (1.1) during 30 min. at r.t.

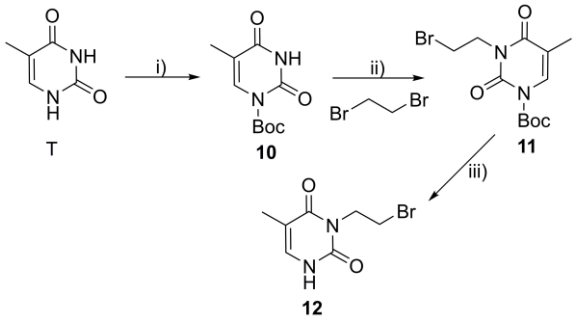
The synthesis of **C** derivative **9** was performed following the method described by Kang *et al*.[27] with some modifications. Briefly, the compound **C** was treated with K_2CO_3 in MeOH in the presence of TBAI as catalyst, followed by the addition of 1,5-dibromopentane **6** to generate the 4-amino-1-(5-bromopentyl)pyrimidin-2(1*H*)-one **9** in 50% yield (Scheme 4). Initially the reaction mixture (**C**, K_2CO_3/TBAI and 1,5-dibromopentane **6**) was stirred at r.t. for 65 h. Then, the thin-layer chromatography (TLC) showed the presence of **C** in the reaction medium so, K_2CO_3/TBAI and 1,5-dibromopentane **6** were added and the mixture was heated at 65 °C for more 24 h (Table 1, entry 14).

The use of the conditions described by Zerdan *et al*.[8] and different phase-transfer catalysts such as TBAA, TBAOH in 40% H_2O or MeOH and C18-crown-6[43] did not afford the *N*-alkylated **C** derivatives (Table 1, entries 10, 11) or led to the formation of **9** in very low yield (Table 1, entries 12-13).



Scheme 4. Synthesis of *N*-alkylated **C** (**9**). I) K_2CO_3, TBAI, MeOH, r.t. to 65 ºC. Yield: 50 % (**9**).

The low yields obtained for **T** alkylation reactions can be explained by the challenging purification process and its poor solubility in common organic solvents. Therefore, the *N*-alkylation of **T** was attempted following other methods described in the literature. One of them, consisted in the one-pot alkylation using primary alcohols such as 3-bromopropanol and 6-bromohexanol.[9] However, none of the methods afforded the envisioned products. In this regard, it has been postulated that the two secondary amines present in the **T** structure contribute to the low regioselectivity observed in several **T** *N*-alkylation reactions.[45,46] Thus, the *N*-protection of one secondary amine was attempted aiming to reduce the reactive sites of the molecule. The 3-(2-bromoethyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione **12** was obtained in three steps: *N*-protection with *tert*-butoxycarbonyl (Boc) protecting group, followed by *N*-alkylation with 1,2-dibromoethane **2** and finally the *N*-deprotection, as illustrated in the Scheme 5. The low overall yield gathered in the synthesis of compound **12** can be explained by the fact that the reactions were incomplete, being necessary to purify the obtained compound in each step by chromatography. The NMR spectra allowed to confirm the introduction of the Boc group at structure **10** and the presence of 2-bromoethyl at product **11**.



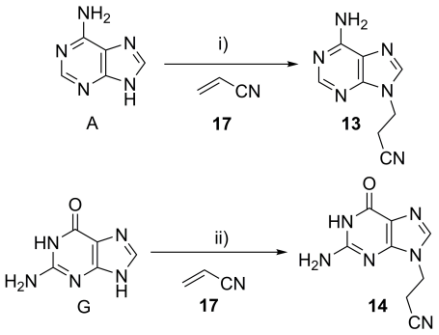
Scheme 5. Synthesis of 3-(2-bromoethyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (**12**). i) Boc_2O, cat. DMAP, MeCN, r.t., 3 h, ii) NaH, dry DMF, 0 ºC to r.t., 30 min, iii) K_2CO_3, MeOH, r.t., 24 h. Yields: 57 % (**10**), 14 % (**11**), 63 % (**12**).

Functionalization of nucleobases via Michael addition reaction

The Michael-type addition reaction has been described in the literature[26,30-32] and involves the addition of the Michael acceptor-

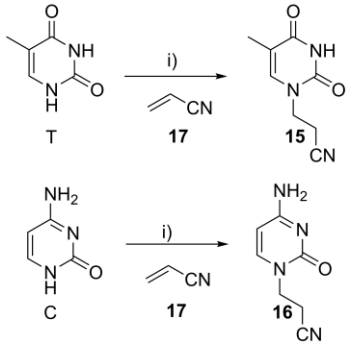
acrylonitrile to a Michael donor **A**, **T** and **C**, usually in the presence of a polar solvent and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or triethylamine (TEA) as deprotonating agent. In this work, the functionalization of all DNA nucleobases with the acrylonitrile group followed the reaction conditions described by Graßl *et al.*[47] with slight modifications, leading to the nucleobase derivatives **13**, **14**, **15** and **16** in 12-35% yields (Table 2).

As highlighted in Table 2, the optimal condition for the synthesis of the compounds **13**, **15** and **16** involves the addition of TBAI to facilitate the nucleobases solubility. **A** was reacted with acrylonitrile **17** in the presence of TBAI under microwave irradiation for 60 min (Table 2, entry 1) but the expected compound **13** was not isolated. However, after conventional heating at 70 ºC for a longer reaction time, **13** was obtained in 12% yield (Table 2, entry 2). Following similar conditions, the reaction of acrylonitrile **17** with **G** did not provide the compound **14** at all or it was obtained in vestigial amounts (Table 2, entries 3, 4 and 5). For the introduction of the propanenitrile group at the secondary amine of **G**, the reaction was performed in slightly basic conditions with the addition of NaOH (2 M) for better solubility, thus being possible the synthesis of 3-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)propanenitrile **14** in modest yield (30%) (Table 2, entry 6). Even using the solution of NaOH (2 M) as base, it was not possible to improve the reaction yield of **14** (Scheme 6).



Scheme 6. Synthesis of *N*-propanenitrile nucleobases (**13**-**14**) by Michael-addition reaction. i) TBAI, MeOH, 70 ºC, ii) TBAI, MeOH, NaOH (2M), 60 ºC, Yields: 12 % (**13**), 30 % (**14**)

Still, using similar conditions, **T** and **C** were successfully modified in modest yield (Table 2, entries 8 and 11). The Michael addition of **T** and **C** were screened using conventional (Table 2, entries 7, 9-10) and MW heating (Table 2, entries 8-9), affording the desired products **15-16** in 20-42% yields. However, using MW irradiation, **15** and **16** were obtained in 40% and 42% yields (Table 2, entries 8 and 11), respectively. The synthesis of the compound **16** was obtained in low yield (Table 2, entry 9) without TBAI at 55 ºC (Scheme 7). We hypothesize that the low to modest yields of the Michael addition reactions could be assigned to the weak nucleophilic nature of the nucleobases, their general difficult manipulation/purification and absence of base in the reaction medium. Nevertheless, the desired compounds **13**-**16** were obtained without the use of DBU or TEA.



Scheme 7. Synthesis of *N*-propanenitrile nucleobases (**15**-**16**) by Michael-addition reaction: (i) TBAI, MeOH, 70 °C. Yields: 33 % (**15**), 35 % (**16**).

Table 2. Screening of the reaction conditions for the synthesis of *N*-propanenitrile nucleobases by Michael addition reaction.

Entry	Nucleobase	Solvent	*T*	Yield (%)
1[a]	**A**	MeOH	MW (60 °C, 60 min.)	-
2[a]	**A**	MeOH	70 °C, 65 h	12
3	**G**	MeOH	55 °C, 89 h	-
4	**G**	MeOH	70 °C, 65 h	traces
5[a]	**G**	MeOH	MW (100 °C, 30 min.)	traces
6[a],[b]	**G**	MeOH	60 °C, 48.5 h	30
7[a]	**T**	MeOH	70 °C, 72 h	33
8[a]	**T**	MeOH	MW (60 °C, 60 min.)	40
9	**C**	MeOH	55 °C, 89 h	20
10[a]	**C**	MeOH	70 °C, 65 h	35
11[a]	**C**	MeOH	MW (60 °C, 60 min.)	42

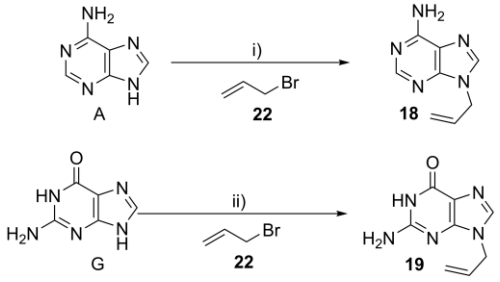
[a] Catalytic amount of TBAI was used as phase-transfer catalyst. [b] NaOH 2 M (40 drops) was used as base.

Functionalization of nucleobases via *N*-allylation and *N*-propargylation reactions

The regioselective synthesis of *N*-allyl and *N*-propargyl **A** and **T**, using commercially available allyl bromide **22** and propargyl bromide **27**, have been described in the literature.[23,24,28,48-49] In general, the products were obtained in low-to-high yields (25-89%) typically using conventional heating overnight or for more than one day. The *N*-allylation of **T** was also described involving two reaction steps, first heating at 65 ºC for 30 minutes followed by MW irradiation for 2 minutes.[29]

In this work, the treatment of **A**, **T**, **C** and **G** with allyl bromide **22**, in basic conditions under MW, led to the desired *N*-allylic pyrimidine and purine derivatives **18**-**21** in low to moderate yields

(16-43%, Table 3) in a shorter reaction time. Moreover, the introduction of the propargyl terminal in **A**, **T** and **C** was also done in basic conditions in modest to high yields 25-61% (Table 4) in reaction times lower than those described in the literature. Changing the reaction conditions of *N*-allylation by resorting to classical heating did not result in the desired products. It is interesting to note that the reaction conditions for the synthesis of each nucleobase derivative changed slightly. For instance, in the case of **A** (Table 3, entries 1-2) there was the need to reduce the amount of allyl bromide **22** (from 3.5 to 1 equiv) to obtain the desired product **18**. Moreover, the increase in the MW irradiation time did not improve the reaction yield. Initial attempts to couple allyl bromide **22** with **G** (Table 3, entry 3) were not successful (only the starting material was recovered). The 9-allyl-2-amino-1,9-dihydro-6*H*-purin-6-one **19** (Table 3, entry 4) was obtained in low yield (26%) using MW in the first reaction step that consisted in the deprotonation of **G** followed by stirring at r.t. during the second step (Scheme 8).



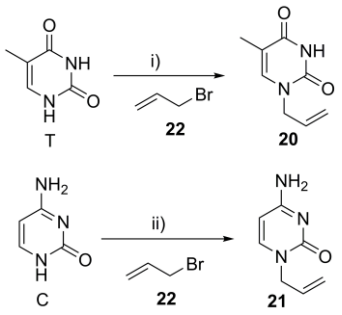
Scheme 8. Synthesis of *N*-allyl nucleobases (**18**-**19**): i) H_2O/NaOH (2M), MW (40 °C, 15 min); ii) H_2O/NaOH (2M), MW (40 °C, 5 min), then r.t. for 3 h. Yields: 16 % (**18**), 26 % (**19**).

Table 3. Screening of the reaction conditions for the synthesis of *N*-allyl nucleobases.

Entry	Nucleobase	*T*	Yield (%)
1[a]	**A**	MW (40 °C, 5 min.), then addition of **22** 40°C, 35 min.	Traces
2[a]	**A**	MW (40 °C, 5 min.), then addition of **22** 40°C, 10 min.	16
3[a]	**G**	MW (40 °C, 5 min), then addition of **22** 40°C, 30 min.	-
4[a]	**G**	MW (40 °C, 5 min.), then addition of **22** stirring under r.t. for 3h	26
5[a]	**T**	MW (40 °C, 5 min.), then addition of **22** 40°C, 35 min.	Traces
6[a]	**T**	MW (40 °C, 5 min.), then addition of **22** 40°C, 10 min.	20
7[a]	**C**	MW (40 °C, 5 min.), then addition of **22** 40°C, 15 min.	Traces
8[a]	**C**	MW (40 °C, 5 min.), then addition of **22** 40°C, 35 min.	43

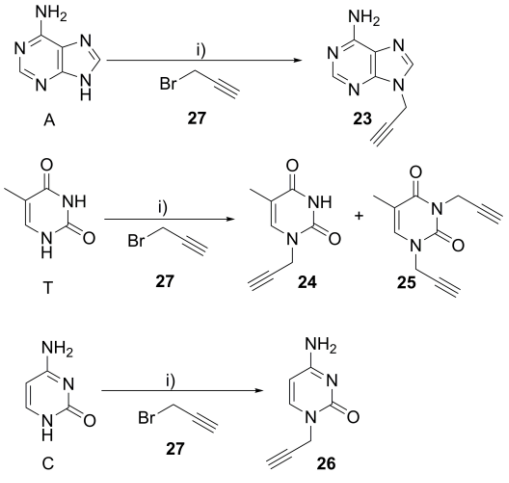
[a] Mixture of H_2O/NaOH 2 M was used as solvent.

The allylation of **T** followed the same reaction conditions used for **A** allylation (Table 3, entries 5-6). As for **C**, the reaction conditions (Table 3, entries 7-8) allowed its chemical functionalization in 43% yield using 3.5 equiv of allyl bromide **22** and longer irradiation time (Scheme 9). This chemical modification was improved significantly under MW conditions and a fast and environmentally friendly method was developed.



Scheme 9. Synthesis of *N*-allyl nucleobases (**20**-**21**). i) H_2O/NaOH (2M), MW (40 °C, 15 min). ii) H_2O/NaOH (2M), MW (40 °C, 40 min). Yields: 20 % (**20**), 43% (**21**).

The *N*-propargylated nucleobases **23**-**26** were obtained following the procedures described elsewhere,[23,24,48-50] with some modifications. The **A**, **T** and **C** were initially treated with NaH in dry DMF, followed by the addition of propargyl bromide **27** at r.t. to generate the products in modest to high yield (25-63%) (Table 4). The compound **23** was obtained in a modest yield (25-30%; Table 4, entries 1-2), probably due to the inherent loss of compound during the purification process by recrystallization. The analysis of the reaction conditions (Table 4, entries 2-3) screened for the synthesis of **T** derivative **24** showed that a decrease of the amount of base and of the reaction time after the addition of propargyl bromide **27** led to the formation of the desired compound as main product in moderate yield (41%). Nevertheless, the N(1) and N(3) bis-adduct **25** was also isolated as a reaction product in 30% yield (Scheme 10).



Scheme 10. Synthesis of *N*-propargyl nucleobases (**23**-**26**). i) dry DMF, NaH 60 % mineral oil, r.t. Yields: 25 % (**23**), 41 % (**24**), 63 % (**26**).

The propargylation of **C** was carried out using K_2CO_3 in MeOH, leading to the compound **26** in low yield (20%; Table 4, entry 4). Subsequently, dry DMF, NaH and **C** were mixed and stirred at r.t. Then, the propargyl bromide **27** was added and the reaction

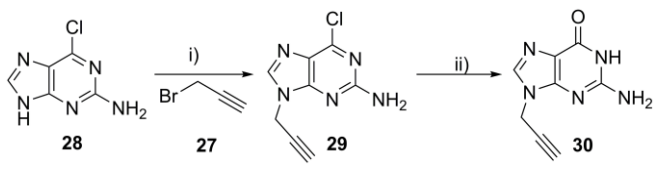
mixture was stirred for 6.5 h affording **26** in modest to good yield (34-63%; Table 4, entries 5-6), respectively (Scheme 10).

Table 4. Screening of the reaction conditions for the synthesis of *N*-propargyl nucleobases.

Entry	Nucleobase	*T*	Base (equiv)	Yield (%)
1[a]	**A**	r.t., 45 min then addition of **27** (1.5) 4 h	NaH[b] (2.0)	25
2[a]	**A**	r.t., 60 min then addition of **27** (1.5) 1.5 h	NaH[b] (2.0)	30
2[a]	**T**	r.t., 45 min then addition of **27** (1.5), 4h	NaH[b] (2.0)	Traces
3[a]	**T**	r.t., 45 min then addition of **27** (1.5), 3 h	NaH[b] (1.5)	41
4[c]	**C**	r.t., 4 days	K_2CO_3 (2.0)	20
5[a]	**C**	r.t., 60 min then addition of **27** (1), 5 h	NaH[b] (2.0)	34
6[a]	**C**	r.t., 40 min then addition of **27** (2), 1.5 h	NaH[b] (2.5)	63
7[a]	**G**	r.t., 3 hours then addition of **27** (1), 3 h	NaH[b] (2.0)	-
8[a]	**G**	r.t., 30 min; 40 °C, 60 min finally addition of **27** (2), 2 h	NaH[b] (2.5)	-
9	**G**	MW (40 °C, 5 min.), then addition of **27** 40°C, 35 min.	NaOH 2M /H_2O (4:1)	traces

[a] DMF was used as solvent. [b] NaH in 60% wt mineral oil. [c] MeOH was used as solvent and TBAI (0.1 equiv) as catalyst.

It is noticeable that the reaction time before and after the addition of propargyl bromide **27** played a key role in this protocol leading to an improvement in the yield for almost the double. Unfortunately, the attempts to generate *N*-propargyl **G** using the reaction conditions tested with **A**, **T** and **C** were not successful (Table 4, entries 7-8) and attempts to do the propargylation under MW irradiation and using NaOH 2M as base only provided the compound **30** in vestigial amounts (Table 4, entry 9). To circumvent the challenges associated to the manipulation of **G**, a protected **G** derivative was used, namely 4-amino-6-chloropurine **28**. The desired compound **30** was obtained using the method described by Nagapradeep *et al.*[51] in 65% overall yield (Scheme 11).



Scheme 11. Synthesis of **G** containing *N*-propargyl terminal (**30**). i) K_2CO_3, dry DMF, r.t., 48 h, ii) TFA:H_2O (3:1), r.t., 48 h. Yields: 50 % (**29**), 80 % (**30**).

In summary, the synthesis of *N*-modified **A** led to the compounds **1** and **3** in high yield (53-77%) and compounds **13**, **18** and **23** in low yield (12-25%). The lower yield of the later compounds is due to the required purifications by recrystallization and column chromatography. For the majority of the reactions with **T**, the regioselective modification of N(1) position was achieved in modest yield (20-33%). However, no product resulting from the N(3) addition or both N(1) and N(3) additions was isolated in either the *N*-alkylation, Michael addition reaction or *N*-allylation. The purification process was time-consuming and laborious, suggesting that eventually other by-products were formed. However, it was not possible to isolate and quantify them. On the other hand, when the regioselectivity of the reaction was controlled using Boc, the overall yield of the compound **12** (45%) was improved and when it was possible to isolate the bis-adduct **25** the overall yield of the *N*-propargylation reaction was 71% yield. The chemical modifications on **C** gave the desired products **9**, **16**, **21** and **26** in moderate to high yields (35-63%), thus confirming that the developed method is regioselective, since the modification occurred only in the N(1) position. The chemical modification of **G** was not successful regarding the alkylation and propargylation, being necessary the use of 2-amino-6-chloropurine **28** as **G** surrogates to allow facile N(9)-propargylation. Indeed, the chemical modification on compound **28** is well-known[8,28,51] due to the difficult chemical manipulation of **G**. In this work, the acrylonitrile and allyl moieties were directly introduced in **G**, without the need to use **G** surrogate **28**, affording compounds **14** and **19** in modest yield (26-30%).

NMR Spectroscopy

The unequivocal structure elucidation of the nucleobase derivatives **1**, **3**, **8**, **9**, **12**, **13**-**16**, **18**-**21**, **23**-**26** and **30** was performed by 1D (^{1}H and ^{13}C) and 2D (HSQC, HMBC, NOESY) NMR (more info on the ESI†). The typical protons in the ^{1}H NMR spectra of *N*-alkylated **A 1** and **3** (see Figures S1 and S3 on the ESI†) are the singlets in the aromatic region corresponding to the resonance of protons 4-NH_2, H-2 and H-8 at δ_H 7.28 ppm, 8.15-8.43 ppm and 8.18-8.70 ppm, respectively. In the aliphatic region, it was possible to identify the signals corresponding to the protons of the bromoethyl and bromopropyl chains, respectively. The aliphatic protons and carbons appear at δ_H 2.35–4.57 ppm (δ_C 20.5-44.6 ppm): as triplets at δ_H 4.40-4.57 ppm (δ_C 44.5-44.6 ppm) and 3.95–4.32 ppm (δ_C 31.6-41.4 ppm) assigned to the NCH_2, BrCH_2, and as a quintet at δ_H 2.35 ppm (δ_C 20.5 ppm) attributed to 2'-CH_2- of the bromopropyl chain. The ^{1}H NMR spectrum of compound **8** (see Figure S5 on the ESI†) showcase the singlet corresponding to the proton resonances of methyl (CH_3) group, at δ_H 1.93 ppm, the quartet at δ_H 6.98 ppm corresponding to proton H-6 with a coupling constant of J = 1.2 Hz due to the coupling with the methyl group 5-CH_3, and the broad singlet at δ_H 8.45 ppm due to the N*H* proton resonance. In the aliphatic region it was possible to see the signals corresponding to the protons of the alkyl chain. The high frequency chemical shift of protons NCH_2 and BrCH_2 at δ_H 3.40-3.73 ppm is due to the deshielding effect of the nitrogen and bromo atoms. The ^{1}H NMR spectrum of compound **12** (see Figure S7 on the ESI†) shows two triplets in the aliphatic region. Such triplets are assigned to the methylene groups bonded to N(3) of **T** that appear at high chemical shift, δ_H 4.16 and 4.79 ppm, due to the deshielding effect of the nitrogen and bromo atoms and the carbonyl groups of C-2

and C-4 positions. The ^{1}H NMR spectrum of *N*-alkylated cytosine **9** (see Figure S9 on the ESI†) presents similar signals of *N*-alkylated **A** and **T** in the aliphatic region. In the aromatic region, two typical doublets were denoted at δ_H 5.83 ppm and 7.80 ppm, being assigned to protons H-5 and H-6, respectively.

The full characterization of the compounds **13**-**16** having a propanenitrile moiety allowed the identification of their main signals. The ^{1}H NMR spectra (see Figures S12, S14, S16 and S18 on the ESI†)) showed two triplets in the aliphatic region corresponding to protons H-1' and H-2' at δ_H 3.88-4.44 ppm and 2.88-3.17 ppm, respectively, as well as the signals characteristic of the purine and pyrimidine rings in the aromatic region. The analysis of the ^{13}C NMR spectrum of each compound was possible with the aid of the 2D NMR spectra (HSQC and HMBC), allowing the assignments of all protonated and quaternary carbon resonances (see Figures S13, S15, S17 and S19 on the ESI†). The connectivities found in the HMBC spectra for H-1' played an important role in the unequivocal assignment of the resonances of the quaternary carbon C-6 and of C-2 and C-4 (in the case of modified **A** and **G**, respectively) and C-2 (in the case of modified **T** and **C**).

The analysis of the ^{1}H NMR spectra (see Figures S20, S22, S24 and S26 on the ESI†) of compounds **18**-**21** enabled the assignment of the chemical shifts of the protons of the allylic system. The signals of compounds **18**, **20** and **21** appear as double triplet (dt) at δ_H 4.83 ppm, 4.34 ppm and 4.26 ppm, with two coupling constants of 1.3-1.6 Hz and 5.4-5.8 Hz assigned to H-1', whereas a multiplet at δ_H 5.66-6.10 ppm was assigned to H-2'. The protons H-3' present different splitting pattern due to the signals overlap of the vinylic system protons. The chemical shift of H-3' of the compounds **18** and **21** appears at δ_H 5.06-5.33 ppm as two doublets of quartets with *cis* coupling $J_{H3'\_H2'}$ = 10.3 Hz, *trans* coupling $J_{H\text{-}3'\_H\text{-}2'}$ = 17.2 Hz and the geminal and allyl couplings $J_{H\text{-}3'\_H\text{-}3'}$ and $J_{H\text{-}3'\_H\text{-}1'}$ = 1.6 Hz. The chemical shift of H-3' of the compounds **19** and **20** appears at δ_H 5.03-5.26 ppm as two doublets of doublets with *trans* coupling $J_{H3'\_H2'}$ = 17.0 Hz, *cis* coupling $J_{H3'\_H2'}$ = 10.4 Hz and the geminal and allyl couplings $J_{H3'\_H3'}$ and $J_{H3'\_H1'}$ = 1.5 Hz. The compound **19** presents a doublet in the aliphatic region at δ_H 4.83 ppm and a multiplet in the aromatic region at δ_H 5.99-6.12 ppm corresponding to H-1' and H-2'.

The connectivities found in the HMBC spectra of compounds **18**-**21** allowed the unequivocal assignment of the quaternary carbon resonances and confirmation of protonated carbons (see Figures S21, S23, S25, S27 on the ESI†), which were assigned from the correlations found in their HSQC spectra. The connectivities found in the HMBC spectra for H-1' allowed the correct assignment of the resonances of C-6 (in the case of modified **A** and **G**) and C-2 (in the case of modified **T** and **C**).

The characteristic signals in the aromatic region in the ^{1}H NMR spectra of *N*-propargyl nucleobases **23**-**24** and **26** (see Figures S28, S30 and S34 on the ESI†) correspond to protons of the purine or pyrimidine structures. Moreover, it is also clear the presence of a triplet at δ_H 3.34-3.47 ppm and a doublet at δ_H 4.48-5.03 ppm, corresponding to the resonances of the protons of the propargyl group. The ^{1}H NMR spectrum of compound **25** (see Figure S32 on the ESI†) shows two signals as triplets at δ_H 3.12 ppm and 3.45 ppm corresponding to protons H-3'' and H-3' of the propargyl chain, respectively, indicating that the modification occurred in both of the **T** N*H* groups. Moreover, one can also denote a doublet of doublets at δ_H 4.56 ppm corresponding to the protons H-1',1'' of the propargyl chain. The connectivities found in the 2D NMR spectra allowed the total assignments of all protonated and quaternary carbon resonances (see Figures S29, S31, S33 and S35 on the ESI†).

Cell viability study

The *in vitro* cytotoxicity of the library of *N*-functionalized nucleobases (**1**, **8**, **9**, **13**-**16**, **18**-**21**, **23**, **24**, **26**, **30**) and native nucleobases (**A**, **T** and **C**) was evaluated using primary human dermal fibroblast cells (HF) by measuring the changes in the metabolic activity of the cells through a resazurin-based assay. The *in vitro* cytotoxicity of the non-functionalized **G** could not be studied due to its poor solubility in both culture media and DMSO. Due to the presence of active metabolites on the viable cells, they can convert the blue-nonfluorescent dye (resazurin) to a pink-fluorescent dye (resorufin), which is proportional to the number of viable cells over a wide concentration range. We started the cytotoxicity studies using a 10 nM–100 µM concentration curve with a dilution factor of 10 (10 nM, 100 nM, 1 µM, 10 µM, 100 µM). The metabolic activity of HF incubated for 72 h with the compounds was measured and compared with the one of compound-free cells (control). All tested compounds exhibited no significant effects at the studied concentration range. The viability index presented values higher than 80%, thus demonstrating that these compounds had no significant effect in the cells' metabolic activity. Besides, the obtained results indicated that, independently of the solution concentration used, no statistically significant differences in cell viability were observed, denoting the excellent biocompatibility of the native and functionalized nucleobase derivatives (Figure 2).

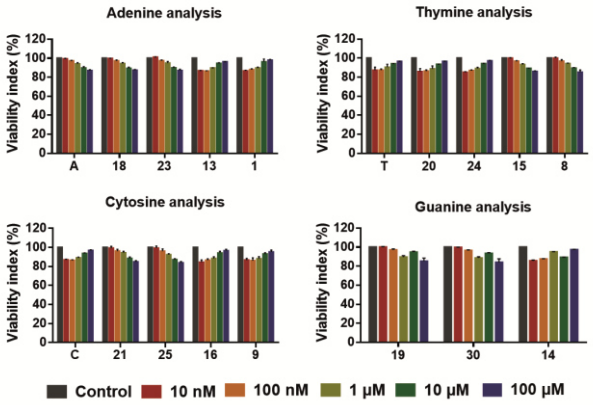


Figure 2. Cell viability analysis of native and functionalized nucleobases at a concentration range between 10 nM-100 µM. Data is presented as mean ± standard deviation of three independent experiments performed in quadruplicate.

It is noteworthy that dermal fibroblasts are the main cell types implicated in the extracellular matrix (ECM) production during the wound healing process and these preliminary cell viability data suggest that the clinical use of these compounds might not have negative effects in the metabolic activity of human fibroblast cells. This is a very important feature for biomedical applications, namely for wound tissue regeneration. Moreover, the nucleobase derivatives can be further functionalized with cell signaling

moieties, including fibronectin or peptide-mimetic proteins to prompt cell adhesion, proliferation, and differentiation. As previously highlighted, the library of functionalized DNA nucleobase derivatives holds great promise for being coupled to a wide array of biomolecules, including polysaccharides or peptides thus potentially leading to innovative high-added value supramolecular biofunctional nanomaterials for biomedicine. However, the *in vitro* biological performance of such innovative biomaterials should be studied to undoubtedly comment on their suitability to address biomedical applications.

Conclusion

The DNA nucleobases (**A**, **T**, **C** and **G**) are important natural heterocyclic building blocks for the rational design and development of advanced and modular soft supramolecular functional nanobiomaterials to be used in the biomedical arena owing to their unique features. In this work, synthetic procedures have been developed for the regioselective functionalization of the secondary amine of the DNA nucleobases with more reactive functional groups in a simple, one-pot and modular manner towards the creation of a library of DNA nucleobase derivatives. The proposed synthetic procedures allowed anchoring distinct chemical functionalities, including *N*-terminal alkyl, *N*-propanenitrile, *N*-allyl, and *N*-propargyl functional groups into the DNA nucleobases, thus extending their functionalities. The nucleobase derivatives were regioselectively synthesized, in most of the cases in a single step, without resorting to the use of protecting groups and subsequent deprotection, thus avoiding two time-consuming steps that usually involve the use of toxic or volatile organic solvents and generate waste. The low cytotoxicity assigned to the toolbox of *N*-functionalized nucleobase derivatives, as well as their intrinsic ability to maintain the high-fidelity molecular recognition capability and structural programmability encoded by the complementary Watson-Crick base pairing open new avenues in the biomedical field. In particular, the library of nucleobase derivatives holds great promise as a highly versatile platform to obtain soft biofunctional materials and complex systems denoting emergent properties and multifunctionalities. For instance, the toolbox of nucleobase derivatives can be coupled to and precisely positioning a wide array of naturally occurring building blocks. Those include peptides, lipids, and carbohydrates, thus possibly enabling the bottom-up assembly of sophisticated and modular supramolecular nanoarchitectures, including adaptive and bioinstructive nanobiomaterials, smart systems, and high-toughness hydrogels that could recreate the structural and functional features of the native ECM of tissues and organs, and be potentially used in controlled drug/therapeutics delivery, biosensing, bioimaging, and tissue engineering strategies.

Experimental Section

Materials and methods

All reagents were commercially available and used without further purification. Column chromatography was performed on silica gel 60, Merck 0.032–0.063 mm and 0.063–0.200 mm, aluminum oxide 90, Alox N, neutral and preparative TLC on silica gel 60 GF_{254} from Merck, and the spots were detected with UV light (254 and 365 nm). The solvents were commercially available first grade. Melting points were obtained using a Büchi melting point B-540 apparatus and are uncorrected. Microwave-assisted reactions were performed in an Ethos MicroSYNTH Labstation (Milestone Inc) using an infrared sensor to control the temperature. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300 or 500 [300.13 MHz (^{1}H), 75.47 MHz (^{13}C) or 500.13 MHz (^{1}H), 125.76 MHz (^{13}C)] spectrometers with tetramethylsilane (TMS) as internal reference. Unequivocal ^{1}H assignments were made with the aid of 2D COSY (^{1}H /^{1}H), whereas ^{13}C assignments were made based on 2D HSQC (^{1}H/^{13}C) and HMBC experiments (delays for one-bond and long-range $J_{C\text{-}H}$ couplings of 147 and 7 Hz). The chemical shifts are reported as δ values (ppm) and coupling constants (J) in Hz. High-mass-resolving ESI-MS were conducted in a Q-Exactive® hybrid quadrupole Orbitrap® mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The instrument was operated in positive mode, with a spray voltage at 3.0 kV and interfaced with a HESI II ion source. The analysis was performed through direct infusion of the prepared solutions at a flow rate of 10 µL min^{-1} into the ESI source, and the operating conditions were as follows: sheath gas (nitrogen) flow rate 5 (arbitrary units); auxiliary gas (nitrogen) 1 (arbitrary units); capillary temperature 320 °C, and S-lens rf level 50. Spectra were analysed using the acquisition software Xcalibur ver. 4.0 (Thermo Scientific, San Jose, CA, USA).

A, **T**, **C** and **G** were obtained from Alfa Aesar. 2-Amino-6-chloropurine, potassium carbonate (K_2CO_3), tetrabutylammonium iodide (TBAI), tetrabutylammonium bromide (TBAB), tetrabutylammonium acetate (TBAA), tetrabutylammonium hydroxide (TBAOH), acrylonitrile, propargyl bromide were purchased from Aldrich and Merck. *N*,*N*-Dimethylformamide (DMF) was purchased from Aldrich and dried with molecular sieves (4 Å) prior to use. All other chemicals were used as received without further purification.

Synthetic procedures

9-(2-bromoethyl)-9*H*-purin-6-amine **1** and 9-(3-bromopropyl)-9*H*-purin-6-amine **3** were synthesized following a procedure described by Srivastava *et al.*[40] and were obtained in good yields [**1**, (900 mg) 53%; **3**, (980 mg) 77%].

The structural characterization of 9-(2-bromoethyl)-9*H*-purin-6-amine **1**, is already reported in the literature[40] and therefore it will not be presented herein.

9-(3-bromopropyl)-9*H*-purin-6-amine (**3**), white solid, m.p. 353-354 °C. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 2.35 (quint, 2H, H-2', J 5.6 Hz); 4.32 (t, 2H, H-3', J 5.6 Hz); 4.70 (t, 2H, H-1', J 5.6 Hz); 8.43 (s, 1H, H-2); 8.70 (s, 1H, H-8) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 20.5 (C-2'); 41.4 (C-3'); 44.5 (C-1'); 116.8 (C-4); 138.3 (C-5); 141.3 (C-8); 145.8 (C-2); 156.4 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 176.1 [((M-HBr)$^+$H)$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_{10}N_5$ ((M-HBr)$^+$H)$^+$, 176.0931; found: 176.0933.

3-(2-Bromoethyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione **12** was synthesized following the procedure described by Jacobsen *et al.*[45] and was obtained in good yield (700 mg) 63%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 1.86 (d, 3H, 5-CH_3, J 1.1 Hz); 4.16 (t, 2H, H-1', J 8.5 Hz); 4.68 (t, 2H, H-2', J 8.5 Hz); 7.58 (q, 1H, H-6, J 1.1 Hz) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 12.2 (5-CH$_3$); 42.4 (C-1'); 66.7 (C-2'); 115.3 (C-5); 150.8 (C-6); 158.8 (C-4); 160.1 (C-2) ppm. MS (ESI$^+$) *m/z* (%): 153.1 [(M+H)$^+$, 100].

Synthesis of 1-(5-bromopentyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (**8**)

A mixture of **T** (146 mg, 1.16 mmol), TBAI (25 mg, 0.069 mmol), K_2CO_3 (160 mg, 1.16 mmol) in dry DMF (8 mL) was stirred at r.t. for 30 min and then 1,5-dibromopentane **6** (266 µL, 1.97 mmol) was added. After 30 h of reaction, at r.t. under nitrogen atmosphere, the unreacted thymine was filtered off, the solvent was evaporated, and the crude purified by column

chromatography using aluminium oxide grade III and a mixture of dichloromethane: hexane (6:4) as eluent. White solid, (54 mg) 20%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 1.45-1.55 (m, 2H, H-4'); 1.67-1.77 (m, 2H, H-2'); 1.82-1.91 (m, 2H, H-3'); 1.93 (s, 3H, 5-CH_3); 3.42 (t, 2H, H-5', *J* 6.6 Hz); 3.71 (t, 2H, H-1', *J* 6.6 Hz); 6.98 (q, 1H, H-6, *J* 1.2 Hz); 8.45 (br s, 1H, 3-N*H*) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 12.6 (5-*C*H$_3$); 24.9 (C-2'); 28.2 (C-3'); 32.0 (C-4'); 33.3 (C-5'); 48.3 (C-1'); 110.7 (C-5); 110.3 (C-6); 150.7 (C-2); 163.9 (C-4) ppm.

Synthesis of 4-amino-1-(5-bromopentyl)pyrimidin-2(1*H*)-one (**9**)

A mixture of **C** (162 mg, 1.46 mmol), TBAI (54 mg, 0.146 mmol), K_2CO_3 (403 mg, 2.92 mmol) in MeOH (10 ml) was stirred at r.t. for 40 min and then 1,5-dibromopentane **6** (335 µL, 2.48 mmol) was added. After 65 h of reaction, at r.t. under nitrogen atmosphere, the starting material was observed by TLC. At that time, K_2CO_3 (202 mg, 1.46 mmol) and 1,5-dibromopentane (335 µL, 2.48 mmol) were added and the mixture was heated at 65 °C for more 24 hours. After this period, methanol was evaporated under vacuum and the crude was purified by column chromatography on alumina grade III, initially using a mixture of 5% of methanol in dichloromethane and finally 20% of methanol in dichloromethane as eluent. Yellow solid, mp 262-263 °C, (180 mg) 50%. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ = 1.33-1.65 (m, 6H, H-2',3',4'); 3.54 (t, 2H, H-5', *J* 7.0 Hz); 3.69 (t, 2H, H-1', *J* 7.0 Hz); 5.82 (d, 1H, H-5, *J* 7.2 Hz); 7.56 and 8.21 (2 br s, 2H, 2-NH_2); 7.79 (d, 1H, H-6, *J* 7.2 Hz) ppm. ^{13}C NMR (125.76 MHz, DMSO-d_6): δ = 24.4 (C-4'); 27.7 (C-2'); 31.8 (C-3'); 35.0 (C-1'); 48.4 (C-5'); 93.1 (C-5); 146.6 (C-6); 153.7 (C-4); 164.9 (C-2) ppm. MS (ESI$^+$) *m/z* (%): 260.1 [(M + H)$^+$, 15]. HRMS (ESI$^+$) *m/z* calcd for $C_9H_{14}N_3OBr$ (M)$^+$, 260.0393; found: 260.0391.

General procedure for the Michael-type reaction of **A**, **T** and **C**

To a solution of the **A**, **T** and **C** (9 mmol) in MeOH (10 ml) was added TBAI (catalytic amount) and acrylonitrile (3.6 mL, 55 mmol). The mixture was heated at 70°C under nitrogen atmosphere for 65, 72 and 65 hours, respectively. The resulting mixture was evaporated and recrystalized in appropriate mixture of hot ethanol/H_2O (80/20).

3-(6-Amino-9*H*-purin-9-yl)propanenitrile (**13**). White solid, mp 256-257 °C, (203 mg) 12%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 3.17 (t, 2H, H-2', *J* 6.5 Hz); 4.44 (t, 2H, H-1', *J* 6.5 Hz); 7.30 (s, 2H, 6-NH_2); 8.17 (s, 1H, H-2); 8.20 (s, 1H, H-8) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 18.1 (C-2'); 38.8 (C-1'); 118.3 (C-3'); 118.7 (C-5); 140.6 (C-8); 149.4 (C-4); 152.6 (C-2); 156.0 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 189.1 [(M+H)$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_9N_6$ (M+H)$^+$, 189.0883; found: 189.0883.

3-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)yl)propanenitrile (**15**). White solid, mp 196-197 °C, (532 mg) 33%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 1.77 (s, 3H, 5-CH_3); 2.90 (t, 2H, H-2', *J* 6.5 Hz); 3.91 (t, 2H, H-1', *J* 6.5 Hz); 7.58 (q, 1H, H-6, *J* 1.1 Hz); 11.4 (s, 1H, 3-N*H*) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 12.0 (5-*C*H$_3$); 16.8 (C-1'); 43.0 (C-2'); 108.8 (C-3'); 118.4 (C-5); 141.0 (C-6); 150.7 (C-2); 164.2 (C-4) ppm. MS (ESI$^+$) *m/z* (%): 180.1 [(M+H)$^+$ 15]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_{10}O_2N_3$ (M+H)$^+$, 180.0768; found: 180.0767.

3-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)propanenitrile (**16**). White solid, mp 246-248 °C, (517 mg) 35%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 2.88 (t, 2H, H-2', *J* 6.5 Hz); 3.88 (t, 2H, H-1', *J* 6.5 Hz); 5.69 (d, 1H, H-5, *J* 7.2 Hz); 7.10 and 7.18 (2 br s, 2H, 4-NH_2); 7.62 (d, 1H, H-6, *J* 7.2 Hz) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 16.8 (C-2'); 44.8 (C-1'); 93.6 (C-5); 118.6 (C-3'); 145.9 (C-6); 155.5 (C-4); 166.2 (C-2) ppm. MS (ESI$^+$) *m/z* (%): 165.1 [(M+H)$^+$, 80]. HRMS (ESI$^+$) *m/z* calcd for $C_7H_9ON_4$ (M+H)$^+$, 165.0771; found: 165.0773.

Synthesis of 3-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)propanenitrile (**14**) by Michael-type reaction

A solution of **G** (500 mg, 3.31 mmol) in MeOH (10 mL) was stirred at 60 °C for 30 min, then TBAI (catalytic amount), acrylonitrile (1.54 mL, 23.2 mmol) and 40 drops of NaOH 2M were added. The reaction mixture was heated at 60 °C and stirred for more 48 h. The solvent was evaporated under vacuum and the crude was recrystalised in a mixture of hot H_2O/ethanol (20:80). White solid, mp > 410 °C, (202 mg) 30%. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ = 3.17 (t, 2H, H-2', *J* 6.3 Hz); 4.43 (t, 2H, H-1', *J* 6.3 Hz); 6.50 (br s, 2H,2-NH_2); 7.93 (s, 1H, H-8) ppm. ^{13}C NMR (125.76 MHz, DMSO-d_6): δ = 19.5 (C-2'); 42.0 (C-1'); 107.9 (C-5); 118.4 (C-3'); 142.9 (C-8); 155.0 (C-2); 157.0 (C-4); 160.6 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 205.1 [(M+H)$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_9ON_6$ (M+H)$^+$, 205.0832; found: 205.0836.

General procedure for the allylation reaction of **A**, **T** and **C**

A suspension of the appropriate nucleobase **A**, **T** or **C** (0.9 mmol) in H_2O: NaOH 2M (1:1) was irradiated for 5 min at 40 °C (300 W), then allylbromide (0.9 mmol, 1.35 mmol or 3.15 mmol for respectively **A**, **T** and **C**) was added and the mixture was irradiated for more 10 min, for **A** and **T**, and 35 min for **C**, at 40 °C, respectively. After removal of the solvent and excess of allyl bromide, 9-allyl-9*H*-purin-6-amine (**18**) and 1-allyl-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (**20**) were purified by flash chromatography using ethyl acetate and CH_2Cl_2: MeOH (98:2), respectively.[28] 1-Allyl-4-aminopyrimidin-2(1*H*)-one (**21**) was filtered off, the solvent evaporated, and the crude was purified by TLC using a mixture of CH_2Cl_2: methanol: ammonia solution (8: 1.6: 0.4).

9-Allyl-9*H*-purin-6-amine (**18**). White solid, mp 138-141 °C, (252 mg) 16%. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ = 4.83 (dt, 2H, H-1', *J* 1.5 and 5.7 Hz); 5.21 (dq, 1H, H-3', *J* 1.6 and 17.1 Hz) 5.33 (dq, 1H, H-3', *J* 1.6 and 10.1 Hz); 5.79 (br s, 2H, 6-NH_2); 5.98-6.19 (m, 1H, H-2'); 7.82 (s, 1H, H-2); 8.38 (s, 1H, H-8) ppm. ^{13}C NMR (75.47 MHz, $CDCl_3$): δ = 45.8 (C-1'); 119.0 (C-3'); 119.6 (C-5); 131.8 (C-2'); 140.4 (C-8); 150.0 (C-4); 153.1 (C-2); 155.4 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 176.1 ([M+H]$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_{10}N_5$ (M+H)$^+$, 176.0931; found: 176.0932.

1-Allyl-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (**20**). White solid, mp 78-80 °C, (300 mg) 20%. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ = 1.93 (s, 3H, 5-CH_3); 4.34 (dt, 2H, H-1', *J* 1.7 and 5.8 Hz); 5.26 (dd, 1H, H-3', *J* 1.4 and 17.0 Hz); 5.31 (dd, 1H, H-3', *J* 1.4 and 10.4 Hz); 5.81-5.94 (m, 1H, H-2'); 6.98 (q, 1H, H-6, *J* 1.2 Hz); 9.30 (s, 1H, 3-N*H*) ppm. ^{13}C NMR (75.47 MHz, $CDCl_3$): δ = 12.4 (5-*C*H$_3$); 49.8 (C-1'); 111.0 (C-5); 119.2 (C-3'); 131.8 (C-2'); 139.7 (C-6); 151.0 (C-2); 164.4 (C-4) ppm. MS (ESI$^+$) *m/z* (%): 167.1 [(M+H)$^+$, 65]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_{11}O_2N_2$ (M+H)$^+$, 167.0811; found: 167.0815.

1-Allyl-4-aminopyrimidin-2(1*H*)-one (**21**). White solid, mp 240-242 °C, (585 mg) 43%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 4.26 (dt, 2H, H-1', *J* 1.5 and 5.4 Hz); 5.06 (dq, 1H, H-3', *J* 1.5 and 17.2 Hz); 5.17 (dq, 1H, H-3', *J* 1.5 and 10.3 Hz); 5.67 (d, 1H, H-5, *J* 7.2 Hz); 5.83-5.94 (m, 1H, H-2'); 6.99 and 7.11 (2 br s, 1H, 4-NH_2); 7.51 (d, 1H, H-6, *J* 7.2 Hz) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 50.3 (C-1'); 93.6 (C-5); 116.9 (C-3'); 134.1 (C-2'); 145.8 (C-6); 155.6 (C-4); 165.9 (C-2) ppm. MS (ESI$^+$) *m/z* (%): 152.1 ([M+H]$^+$, 100)]. HRMS (ESI$^+$) *m/z* calcd for $C_7H_{10}ON_3$ (M+H)$^+$, 152.0818; found: 152.0816.

Synthesis of 9-allyl-2-amino-1,9-dihydro-6*H*-purin-6-one (**19**) by allylation reaction

A solution of **G** (200 mg, 1.32 mmol) in H_2O:NaOH 2M (1:4) was irradiated for 5 min, at 40 °C (300 W). Then, 3-bromoprop-1-ene **22** (400 µL, 4.63 mmol) was added and the reaction mixture was stirred for 3 h at r.t. and protected from light. The solvent was evaporated, and the mixture was purified by TLC using a mixture of CH_2Cl_2: methanol: ammonia solution (8: 1.4: 0.6). Greyish solid, mp > 410 °C, (66 mg) 26%. ^{1}H NMR (500.13 MHz, DMSO-d_6): δ = 4.83 (d, 2H, H-1', *J* 5.5 Hz); 5.03 (dd, 1H, H-3', *J* 1.4 and 17.1 Hz); 5.17 (dd, 1H, H-3', *J* 1.4 and 10.1 Hz); 5.99-6.10 (m, 1H, H-2'); 6.12 (br s, 2H, 2-NH_2), 7.89 (s, 1H, H-8); 10.76 (br s, 1H, 1-N*H*) ppm. ^{13}C

NMR (125.76 MHz, DMSO-d_6): δ = 48.0 (C-1'); 108.0 (C-5); 117.2 (C-3'); 134.4 (C-2'); 142.9 (C-8); 144.1 (C-2); 149.4 (C-4); 152.8 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 192.1 ([M+H]$^+$, 100].

General procedure for the propargylation reaction of **A**, **T** and **C**

To a solution of the appropriate nucleobase **A**, **T** or **C** (2.7 mmol) in dry DMF (5 mL) was added NaH 60 % mineral oil (5.40, 4.04 and 6.75 mmol for respectively **A**, **T** and **C**) and the mixture was stirred for 45 min, for **A** and **T**, and 40 min for **C**, under nitrogen atmosphere. Then propargyl bromide **27** (4.04, 4.04 and 5.4 mmol for **A**, **T** and **C**, respectively) was added and the reaction was stirred for more 4 h, 3 h and 1.5 h, respectively. 9-(Prop-2-yn-1-yl)-9*H*-purin-6-amine (**23**) was filtered off, the solvent evaporated and the crude product purified through recrystallization (twice from MeOH).[23] 5-Methyl-1-(prop-2-yn-1-yl)pyrimidine-2,4(1*H*,3*H*)-dione (**24**) was purified by column chromatography (CH_2Cl_2; followed by CH_2Cl_2:MeOH, 98:2 and 95:5). 4-Amino-1-(prop-2-yn-1-yl)pyrimidin-2(1*H*)-one (**25**) was evaporated and the crude was recrystalized in hot ethanol.

9-(Prop-2-yn-1-yl)-9*H*-purin-6-amine (**23**). White solid, mp 201-203 °C, (117 mg) 25%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 3.47 (t, 1H, H-3', *J* 2.5 Hz); 5.03 (d, 2H, H-1', *J* 2.5 Hz); 7.29 (br s, 2H, 6-NH_2); 8.17 (s, 1H, H-2); 8.19 (s, 1H, H-8) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 32.2 (C-1'); 75.8 (C-3'); 78.3 (C-2'); 118.5 (C-5); 140.1 (C-8); 149.1 (C-4); 152.7 (C-2); 156.0 (C-6) ppm. MS (ESI$^+$) *m/z* (%): 174.1 [(M+H]$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_8N_5$ (M+H)$^+$, 174.0772; found: 174.0774.

5-Methyl-1-(prop-2-yn-1-yl)pyrimidine-2,4(1*H*,3*H*)-dione (**24**). White solid, mp 157-158 °C, (182 mg) 41%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 1.77 (d, 3H, *J* 0.7 Hz, 5-CH_3); 3.40 (t, 1H, H-3', *J* 2.5 Hz); 4.47 (d, 2H, H-1', *J* 2.5 Hz); 7.57 (q, 1H, H-6, *J* 1.2 Hz); 11.38 (br s, 1H, 3-N*H*) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 11.9 (5-*C*H$_3$); 36.3 (C-1'); 75.7 (C-3'); 78.7 (C-2'); 109.4 (C-5); 140.1 (C-6); 150.4 (C-2); 164.2 (C-4) ppm. MS (ESI$^+$) *m/z* (%): 165.1 ([M+H]$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_8H_9O_2N_2$ (M+H)$^+$, 165.0654; found: 165.0659.

5-Methyl-1,3-di(prop-2-yn-1-yl)pyrimidine-2,4(1*H*,3*H*)-dione (**25**). Yellow solid, (50 mg) 30%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 1.84 (d, 3H, *J* 1.2 Hz, 5-CH_3); 3.12 (t, 1H, H-3'', *J* 2.4 Hz); 3.45 (t, 1H, H-3', *J* 2.4 Hz); 4.56 (dd, 4H, H-1',1'', *J* 2.5 and 4.9 Hz); 7.70 (d, 1H, H-6, *J* 1.2 Hz) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 12.5 (5-*C*H$_3$); 30.1 (C-1''); 37.6 (C-1'); 73.1 (C-3''); 76.1 (C-3'); 78.4 (C-2'); 79.1 (C-2''); 108.6 (C-5); 139.3 (C-6); 149.8 (C-2); 164.1 (C-4) ppm.

4-Amino-1-(prop-2-yn-1-yl)pyrimidin-2(1*H*)-one (**26**). Brownish solid, mp 224-226 °C, (254 mg) 63%. ^{1}H NMR (300.13 MHz, DMSO-d_6): δ = 3.38 (t, 1H, H-3', *J* 2.5 Hz); 4.47 (d, 2H, H-1', *J* 2.5 Hz); 5.71 (d, 1H, H-5, *J* 7.2 Hz); 7.08 and 7.17 (2 br s, 2H, 4-NH_2); 7.64 (d, 1H, H-6, *J* 7.2 Hz) ppm. ^{13}C NMR (75.47 MHz, DMSO-d_6): δ = 37.3 (C-1'); 75.3 (C-3'); 79.5 (C-2'); 94.1 (C-5); 144.8 (C-6); 155.2 (C-4); 166.0 (C-2) ppm. MS (ESI$^+$) *m/z* (%): 150.1 [(M+H)$^+$, 100]. HRMS (ESI$^+$) *m/z* calcd for $C_7H_8N_3$ (M+H)$^+$, 150.0662; found: 150.0662.

The synthesis of 2-amino-9-(prop-2-yn-1-yl)-1,9-dihydro-6*H*-purin-6-one **30** was performed following the procedure described by Nagapradeep *et al.*[51] and **30** was obtained in a very good yield (80%; 441 mg).

Cell viability study

Preparation of solutions. Nucleobases (**A**, **T** and **C**) and functionalized nucleobases (**1**, **8**, **9**, **13**-**16**, **18**-**21**, **23**, **24**, **26**, **30**) were dissolved in 100% DMSO (Sigma-Aldrich) to obtain a stock concentration of 100 mM. Thereafter, dilutions were performed in growth medium. The maximum DMSO concentration applied to the cells was 0.1% (v/v) to avoid toxic effects associated with higher concentrations of this solvent.

Cell culture. A human primary dermal fibroblast cell line (HF) (ATCC PCS-201-012TM) was grown in α-MEM culture medium supplemented with 10% FBS (South America origin, Thermo Fisher Scientific) and 1% penicillin/streptomycin solution (Thermo Fisher Scientific). Cell cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Cell viability assay. Serial dilutions ranged from 10 nM to 100 µM to cover a wide scale for the generation of dose–response curves were prepared from the stock solution (100 mM). In all experiments, the solvent DMSO (0.1% v/v) alone was used as a negative control. The HF were seeded in 96-well plates (final volume 100 µL) at a density of 3.5×10^3 viable cells per cm^2 and incubated for 24 h. Cells were then exposed to the test compounds diluted in culture medium (final volume 200 µL) and 48 hours post transfection, 100 µL of the culture medium was replaced by test compounds for additional 24 hours. Thereafter, cell viability was assessed by using fresh medium containing 10% of resazurin-based alamarBlue™ reagent (100 µL per well (Life Technologies)), according to the manufacturer's instructions. Values considered after 3 hours incubation were within the linear range of the reading and the cell's viability was expressed as a percentage of viability of nontreated cells.

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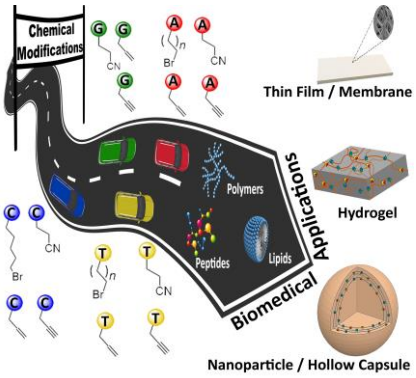

Regioselective *N–H* functionalization of DNA nucleobases (A, T, C, G): One-pot synthetic procedures for producing a library of nucleobase derivatives enlisted with reactive functional groups for bioconjugation and cross-linking reactions with other biomolecules are reported. The nucleobase derivatives bearing either *N*-alkyl, *N*-propanenitrile, *N*-allyl or *N*-propargyl terminals are non-cytotoxic, denoting great promise for biomedical applications.

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