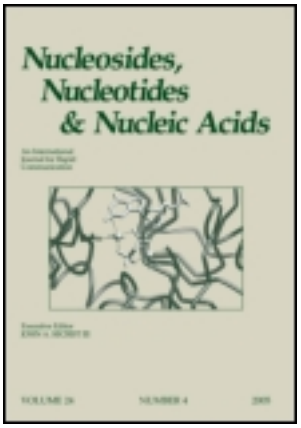

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Mr Mikhail Yu. Steklov [a b], Dr Vitali I. Tararov [a], Professor Georgy A. Romanov [b] & Sergey N. Mikhailov [a]

[a] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

[b] Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia

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FACILE SYNTHESIS OF 8-AZIDO-6-BENZYLAMINOPURINE

Mikhail Yu. Steklov,[1,2] Vitali I. Tararov,[1] Georgy A. Romanov,[2] and Sergey N. Mikhailov[1]
[1]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia*
[2]*Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia*

□ *Bromination of 6-benzylaminopurine (**1**) with Br_2 in AcOH in the presence of AcONa afforded 6-benzylamino-8-bromopurine (**2**) in 59% yield. The position of bromination was confirmed by direct transformation of bromide **2** by reaction with NaN_3 in dimethyl sulfoxide to 8-azido-6-benzylaminopurine (**3**) in a yield of 70% and comparison of its properties with the known compound 2-azido-6-benzylaminopurine (**11**). Compounds **3** and **11** were checked for their biological activity in specific biotests based on the primary cytokinin effects in living plants. Both synthesized compounds displayed effects similar to the typical cytokinin 6-benzylaminopurine (**1**).*

Keywords Cytokinins; photoaffinity labeling; 8-azido-6-benzylaminopurine

INTRODUCTION

Cytokinins constitute a group of plant hormones and related synthetic bioregulators that exert multiform effects on plant growth and development. Endogenous cytokinins stimulate cell division, photomorphogenesis, chloroplast development, and pigment biosynthesis, regulate shoot and root growth and overall plant architecture, and counteract leaf aging and apical dominance.[1,2]

Cytokinins derivatives with the azido group have been used in many photoaffinity-labeling experiments.[3–5] Photolysis of the azido group generates a highly reactive nitrene species that can covalently bind to proteins by insertion in the nearest O-H, N-H, and S-H bonds. This property is used to scrutinize the structure of the cytokinin-binding protein active sites.[6] The modern trend is the application of azido-modified natural compounds for "click-chemistry" widely used in the modification of nucleic acids, material

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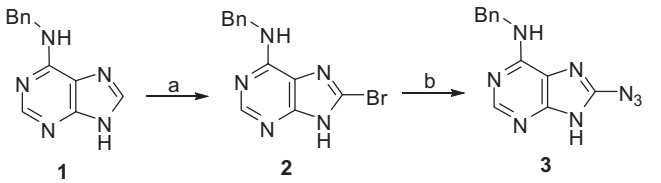
Address correspondence to Sergey N. Mikhailov, Engelhardt Institute of Molecular Biology, Vavilov st 32, Moscow, 119991 Russia. E-mail: smikh@eimb.ru

science, and drug discovery. Several sensitive methods for detection and location of biomolecules in living cells have been developed utilizing this approach.[7–11] Azido-modified cytokinins will be of interest for detection of cytokinin distribution in plants. Currently, a facile two-step preparation of 8-azido-6-benzyaminopurine (**3**) from available 6-benzylaminopurine (**1**) is disclosed.

RESULTS AND DISCUSSION

The simple methods for preparation of azido-modified cytokinins are of great value due to the importance of these substances for biological assays. We were rather interested in obtaining of 8-azido-6-benzylaminopurine (**3**). To our surprise, we could not find in the literature adequate methods of its synthesis. The first reported strategy starts from hardly available 6,8-dichloropurine.[6] The alternative route from adenosine consists of five successive steps: bromination of adenosine in position 8, substitution of bromine with NaN_3, 1-*N*-benzylation with BnBr, subsequent Dimroth rearrangement to 8-azido-6-benzyladenosine, and final cleavage of glycoside bond.[12] The isolated total yield of **3** was 6%. At the same time, the authors claimed that bromination of **1** with Br_2 gave likely 2-bromoderivative, and it was not possible to substitute bromine atom on azido group.[12]

Bromination of **1** in position 2 seemed to be disputable. Indeed, adenosine was successfully brominated with Br_2 in position 8,[13] and the reaction of adenine with Br_2 also gave 8-bromoderivative.[14] It is rather doubtful to expect that N^6-benzyl substituent in adenine moiety should dramatically change the site of Br_2 attack. As soon as the synthesis of 8-azido-6-benzylaminopurine (**3**) via 8-bromo compound **2** is straightforward (Scheme 1), we focused on bromination of 6-benzylaminopurine (**1**). A virtue of this approach is that the photo-sensitive azido group is introduced in the molecule in the final step.



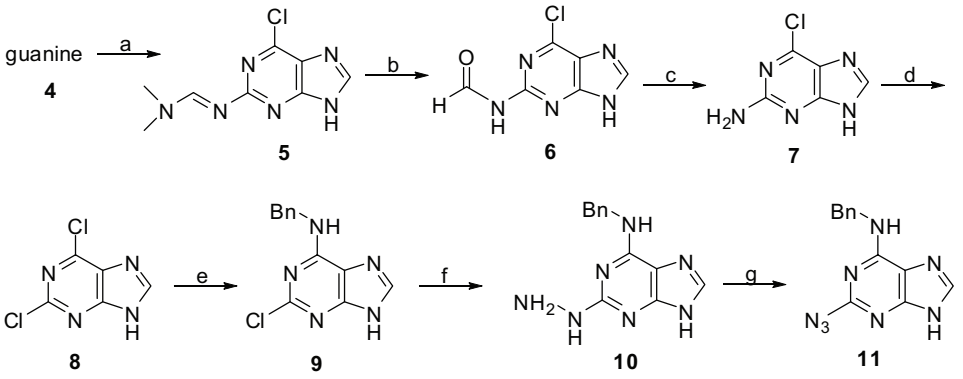
SCHEME 1 Two-step synthesis of 8-azido-6-benzylaminopurine (**3**). Reagents and conditions: a) Br_2, AcOH, AcONa, room temperature, 24 hours, 59%; b) NaN_3, dimethyl sulfoxide (DMSO), 75°C, 36 hours, 70%.

Attempts to utilize the reported methods of bromination of adenine and adenosine in water solution at ambient temperature failed when applied to 6-benzylaminopurine (**1**). The possible reason for it was poor solubility of the starting compound and the product in the reaction media. We have found

out that 6-benzylaminopurine is sufficiently soluble in AcOH. Nevertheless, the reaction of 6-benzylaminopurine with 1 equivalent of Br_2 in AcOH gave only low yield (20%) of the product **3**. Addition of 1 equivalent of AcONa did not improve the situation. When fourfold excess of Br_2 and AcONa was used, the yield of the product increased significantly to 59%. The coarse of the bromination can be monitored by thin layer chromatography (TLC) on silica gel (AcOH-CH_2Cl_2 1:25). Even by applying the excess of reagents, the starting compound remained in the reaction mixture.

The reaction of bromide **2** with threefold excess of NaN_3 in DMSO at 75°C was rather slow. A consecutive 36 hours of heating were required to complete the reaction. The reaction was monitored by ^{1}H NMR spectroscopy as soon as we were unable to find an appropriate developing system for their separation on TLC. Aliquots were taken from the reaction mixture after each 8 hours of heating. The samples were diluted with DMSO-d_6 before running the NMR spectrum. The 2-H resonance peak of the bromide **2** at 8.17 ppm slowly decreased with time, and simultaneously the 2-H peak of the azide **3** at 8.13 ppm increased. Pure azide **3** was isolated in a yield of 70%. Its purity and identity were confirmed by liquid chromatography/mass spectroscopy (LC-MS). Chromatograms showed only one peak with *z/e* 267.06 [M+H$^+$] (or *z/e* 311.02 [M-H$^+$ + HCOOH]), which corresponds to azide **3**.

To prove the structure of the azide **3**, we synthesized 2-azido-6-benzylaminopurine (**11**) according to published methods[15–17] starting from guanine (Scheme 2). This sequence was selected due to availability of guanine, and intermediate compounds could be easily isolated and did not demand extra purification. Moreover, 2,6-dichloropurine (**8**) is a key compound in the synthesis of biologically active nucleosides, reversine, and related compounds.[18–20] We have observed that published procedures are highly reproducible and may be easily scaled up.



SCHEME 2 Synthesis of 2-azido-6-benzylaminopurine (**11**). Reagents and conditions: a) $POCl_3$, dimethylformamide (DMF), 80°C, 8 hours, 72%; b) 12% aqueous AcOH, 70°C, 4.5 hours, 75%; c) 10% aqueous NaOH, room temperature, 3 hours, 89%; d) $NaNO_2$, conc. HCl, $ZnCl_2$, 5°C, 1 hour, 54%; e) benzylamine, isopropanol, reflux, 3.5 hours, 77%; f) 85% hydrazine hydrate in water, reflux, 1.5 hours, 84%; g) $NaNO_2$, HCl, AcOH, room temperature, 96 hours, 82%.

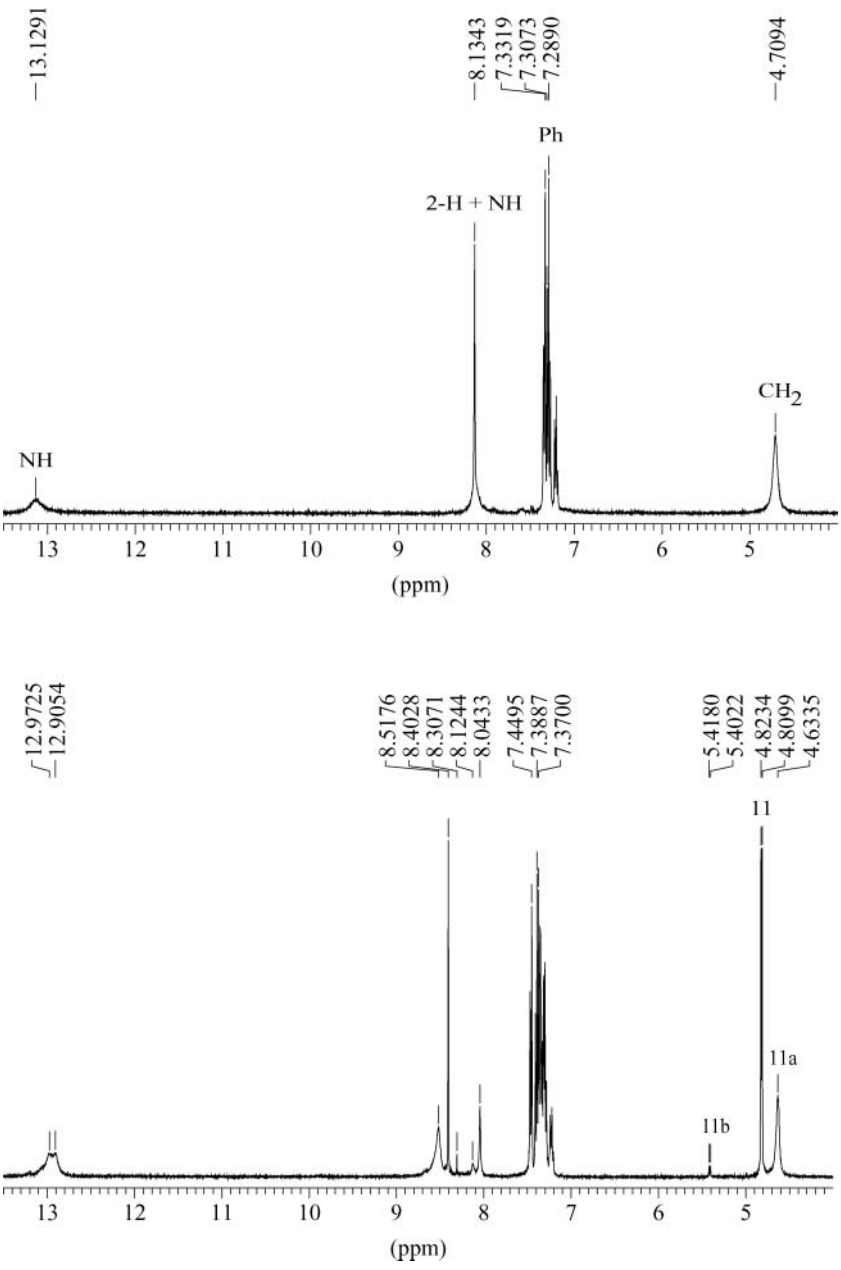
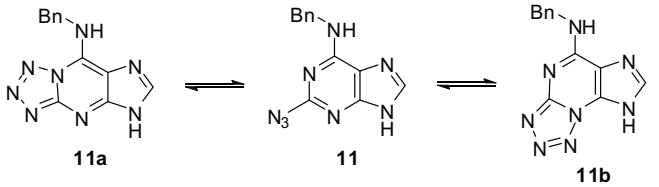


FIGURE 1 The 400 MHz ^{1}H NMR spectra in DMSO-d_6 at 32°C: (top) 8-azido-6-benzyaminopurine (**3**) and (bottom) 2-azido-6-benzylaminopurine (**11**).

The detailed preparation of azide **11** is given in the Experimental part. ^{1}H NMR spectra of azides **3** and **11** are presented in Figure 1. The striking difference between both compounds is evident.

In DMSO-d_6 in the case of the azide **11**, formation of two additional cyclic isomers **11a** and **11b** is observed (Scheme 3). The ratio **11**:**11a**:**11b** is equal



SCHEME 3 Azido-tetrazolo tautomerism of 2-azido-6-benzylaminopurine (**11**).

to 0.56:0.41:0.03 (Figure 1). The rate of interconversion of these species is slow enough to be detected by ^{1}H NMR. Similar tautomeric equilibrium for 2-azidoadenine is well documented.[21] In contrast, the azide **3** at the same conditions displays simple spectrum in which no other isomer could be detected, which are in accordance with the literature data.[12] Furthermore, LC-MS analysis of the azide **11** gives three chromatographic peaks with the same *m/e* 267.06 [M+H$^+$], while only one peak with this *m/e* value was detected in the case of compound **3**.

In conclusion, we have developed a simple and facile two-step method for the preparation of 8-azido-6-benzylaminopurine (**3**) starting from available 6-benzylaminopurine (**1**) with the crucial step being bromination of **1** with Br_2. Compounds **3** and **11** were checked for their biological activity in specific biotests based on the primary cytokinin effects in living plants. Both synthesized compounds displayed effects similar to the typical cytokinin 6-benzylaminopurine (**1**). These findings are of importance for further use of azido derivatives of benzylaminopurine for affinity labeling and "click" reactions.

EXPERIMENTAL

The solvents and materials of reagent grade were used without additional purification. Melting points were determined by an electrothermal apparatus and were uncorrected. TLC was performed on Alugram SIL G/UV254 (Macherey-Nagel) with ultraviolet (UV) visualization. ^{1}H NMR spectra were recorded on a Bruker AMX 400 NMR instrument at 32°C. Chemical shifts in ppm were measured relative to the residual solvent signal as an internal standard (DMSO-d_6, $\delta = 2.50$ ppm). The position of NH resonances was additionally confirmed by their exchange with the addition of D_2O. The UV spectra were recorded by a Cary300UV/VIS spectrophotometer (Varian). LC-MS analysis was performed on Surveyor MSQ instrument (Thermo Finnigan, USA), operating in atmospheric pressure chemical ionization (APCI) mode with the detection of positive and negative ions, and equipped with Onyx Monollithic C18 25 × 4.6 mm Part No CHO-7645 column. The eluent was a gradient of 0.1% HCOOH aqueous solution in MeCN. Chromatographic peaks were detected simultaneously with an evaporative light scattering detector (ELSD), photodiode array detector (PAD), and total ion

current (TIC) detector. In all cases (except the azide **11**), only one peak was revealed and the chromatographic purity of compounds was more than 99%.

Biological Materials and Methods

Cytokinin activity assays were accomplished by using model plant systems with Amaranthus (*Amaranthus caudatus* L.)[22] and transgenic P_{ARR5}*: GUS* Arabidopsis (*Arabidopsis thaliana* L.) seedlings.[23] Both assay systems are specifically sensitive to cytokinins and respond to hormone application in a few hours, making possible quantitative determinations. For a positive control, 6-benzyladenine (BA) was applied.

6-Benzylamino-8-bromopurine (2)

To a stirred solution of the compound **1** (200 mg, 0.888 mmol) and AcONa × $3H_2O$ (534 mg, 3.93 mmol) in AcOH, (5 mL) bromine (0.184 mL, 3.59 mmol) was added in one portion at room temperature. The precipitate began to form after 2 hours. The reaction was controlled by TLC (AcOH/CH_2Cl_2 1:25). After stirring for 24 hours, the precipitate was filtered and washed thoroughly with acetone until it became colorless, yielding after drying over P_2O_5 160 mg (59%) of **2** as a colorless solid. $R_f = 0.53$ (AcOH-CH_2Cl_2, 1:25). Mp 210°C–212°C. ^{1}H NMR (DMSO-d_6): 13.65 (br s, 1H, N-H), 8.47 (br s, 1H, N-H), 8.17 (s, 1H, 2-H), 7.18–7.36 (m, 5H, H-Ph), 4.69 (br s, 2H, CH_2N). UV (H_2O), λ_{max} in nm (ε): pH 2, 211 (14500), 279 (12700); pH 7, 215 (16900), 275 (11600); pH 12, 277 (14200). Anal. Calcd. for $C_{12}H_{10}BrN_5$: C, 47.39; H, 3.31; N, 23.03. Found: C, 47.25; H, 2.14; N, 22.83.

8-Azido-6-benzyaminopurine (3)

A mixture of NaN_3 (543 mg, 8.34 mmol) and bromide **2** (790 mg, 2.61 mmol) in DMSO (11 mL) was heated with stirring at 75°C for 36 hours. The reaction was monitored by ^{1}H NMR (a volume of the reaction mixture containing 1–2 mg of substance was diluted with DMSO-d_6). After cooling at room temperature, the mixture was poured into 200 mL of cold water. The precipitate was filtered and dried over P_2O_5 to give 486 mg (70%) of **3** as a pale yellow solid. $R_f = 0.53$ (AcOH-CH_2Cl_2, 1:25). Mp 170°C–172°C. Lit.: Mp 175°C.[12] ^{1}H NMR (DMSO-d_6): 13.16 (br s, 1H, N-H), 8.13 (br s, 2H, 2-H and N-H), 7.07–7.48 (m, 5H, H-Ph), 4.71(br s, 2H, CH_2N). MS (APCI): *m/z* [M+H^+] calcd. for $C_{12}H_{11}N_8$: 267.26, found 267.06; *m/z* [M-H^- + HCOOH] calcd. for $C_{13}H_{11}N_8O_2$: 311.28, found 311.02. UV (H_2O), λ_{max} in nm (ε): pH 2, 296 (2400); pH 7, 218 (19700), 290 (1800); pH 12, 292 (20400).

6-Chloro-2-formylaminopurine acetate (6)

To a solution of 1,2-dichloroethane (50 mL), DMF (14 mL, 0.18 mol) and $POCl_3$ (8.4 mL, 0.09 mol) guanine (4.53 g, 30 mmol) was added and the mixture was stirred at 80°C for 8 hours. After cooling at room temperature, the reaction mixture was poured into 120 mL of water. The mixture was neutralized to pH 5 by adding sodium carbonate (17.5 g, 0.165 mol). The mixture was stirred for 30 minutes and then was allowed to stand until the partition of layers ceased. The aqueous layer was separated and solid NaOH (2.52 g, 0.063 mol) was slowly added. The precipitated product was filtered, washed with water, and dried over P_2O_5 to give 4.88 g (72%) of **5** as a yellow solid. The compound was used in the next step without additional purification. $R_f = 0.70$ (EtOH-CH_2Cl_2, 1:9). ^{1}H NMR (DMSO-d_6): 13.15 (br s, 1H, N-H), 8.57 (s, 1H, 8-H), 8.29 (s, 1H, N=CH-N), 3.14 (s, 3H, Me), 3.03 (s, 3H, Me).

The compound **5** (4.88 g, 21.7 mmol) was mixed with 12% (*v/v*) AcOH aqueous solution (52 mL) and stirred at 70°C for 4.5 hours. After cooling at 20°C, the precipitate was filtered, washed with water, and dried over P_2O_5 to give 3.2 g (75%) of **6** as a yellow solid. $R_f = 0.75$ (EtOH-CH_2Cl_2, 1:9). Mp > 260°C. ^{1}H NMR (DMSO-d_6): 12.80 (br s, 2H, N-H + AcO<u>H</u>), 11.07 (d, ^3J = 9.6 Hz, 1H, HC=O), 9.32 (d, ^3J = 9.6 Hz, 1H, N-H), 8.48 (s, 1H, 8-H), 1.91 (s, 3H, Ac). UV (H_2O), λ_{max} in nm (ε): pH 2, 225 (20200), 295 (5000); pH 7, 226 (21300), 294 (5400); pH 12, 239 (15500), 273 (3400), 305 (6000).

2-Amino-6-chloropurine (7)

The compound **6** (3.0 g, 15.2 mmol) was dissolved in 10% (*w/w*) NaOH aqueous solution (20 mL) and stirred at room temperature for 3 hours. Then, the reaction solution was neutralized with conc. hydrochloric acid. The resulted precipitate was filtered, washed with water, and dried over P_2O_5 to give 2.3 g (89%) of **7** as a pale yellow solid. $R_f = 0.60$ (EtOH-CH_2Cl_2, 1:9). Mp > 260°C.[16] ^{1}H NMR (DMSO-d_6): 12.81 (br s, 1H, N-H), 8.10 (s, 1H, 8-H), 6.75 (br s, 2H, NH_2). UV (H_2O), λ_{max} in nm (ε): pH 2, 214 (28600), 238 (6600), 314 (7000); pH 7, 216 (29400), 240 (6200), 307 (7000); pH 12, 271 (4100), 308 (6500).

2,6-Dichloropurine (8)

A solution of $ZnCl_2$ (7.0 g, 110 mmol) in conc. hydrochloric acid (9.2 mL) was cooled down at 10°C, and finely powdered compound **7** (2.3 g, 13.6 mmol) was added with stirring. The resulting stirred mixture was cooled further at −5°C and $NaNO_2$ (1.27 g, 18.4 mmol) was added over a period of 30 minutes keeping the temperature below 5°C. The mixture was stirred for additional 30 minutes and was diluted with 12 mL of water. The product was extracted with ethyl acetate (4 × 12 mL). The organic phase was washed with water (2 × 7 mL.), dried over Na_2SO_4, and evaporated to dryness. The residue was recrystallized from MeOH to yield after drying over P_2O_5 1.4 g

(54%) of (**8**) as slightly yellow crystals. $R_f = 0.53$ (EtOH-CH_2Cl_2, 1:25). Mp 178°C–180°C. Lit.: Mp 184°C–186°C.[17] ^{1}H NMR (DMSO-d_6): 14.10 (br s, 1H, N-H), 8.72 (s, 1H, 8-H). UV (H_2O), λ_{max} in nm (ε): pH 2, 210 (23900), 273 (9500); pH 7, 211 (23500), 275 (9300); pH 12, 276 (7800).

6-Benzylamino-2-chloropurine (9)

A mixture of compound **8** (0.6 g, 3.17 mmol), benzylamine (1.22 mL, 11.2 mmol), and 2-propanol (9 mL) was heated at 82°C for 3.5 hours. After cooling, the volatiles were removed under reduced pressure. Water (27 mL) was added to the residue, and the mixture was kept at 0°C for several hours. The precipitated product was collected by filtration, washed well with water, and dried over P_2O_5, yielding 632 mg (77%) of **9** as a colorless solid. $R_f = 0.65$ (EtOH-CH_2Cl_2, 1:25). Mp 239°C–241°C. Lit.: Mp 245°C–246°C.[15] ^{1}H NMR (DMSO-d_6): 12.93 (br s, 1H, N-H), 8.59 (br s, 1H, N-H), 8.13 (s, 1H, 8-H), 7.42–7.18 (m, 5H, Ph), 4.65 (br s, 2H, CH_2N). UV (H_2O), λ_{max} in nm (ε): pH 2, 210 (23500), 274 (16400); pH 7, 210 (24200), 272 (17300); pH 12, 277 (15400).

6-Benzylamino-2-hydrazinopurine (10)

A mixture of compound **9** (600 mg, 2.31 mmol) in 85% solution of hydrazine hydrate in water (9 mL) was stirred and refluxed for 1.5 hours. After cooling to room temperature, a white crystalline precipitate was collected by filtration, washed well with water, and recrystallized from absolute ethanol to give after drying over P_2O_5 492 mg (84%) of **10** as colorless crystals. $R_f = 0.33$ (EtOH-CH_2Cl_2, 1:25). Mp 248°C–250°C. Lit.: Mp 252°C.[15] ^{1}H NMR (DMSO-d_6): 12.26 (br s, 1H, N-H), 7.75 (br s, 1H, N-H), 7.69 (s, 1H, 8-H), 7.09–7.45 (m, 5H, Ph), 4.66 (br s, 2H, CH_2N), 3.98 (br s, 1H, N-H). UV (H_2O), λ_{max} in nm (ε): pH 2, 207, (17400), 245 (9800), 277 (10800); pH 7, 206 (17500), 246 (11200), 275 (11400); pH 12, 284 (11300).

2-Azido-6-benzylaminopurine (11)

To a mixture of compound **10** (480 mg, 1.88 mmol) in 10% (*v/v*) aqueous AcOH solution (12 mL) containing sodium nitrite (133 mg, 1.9 mmol) was added 2 mL of 1 N HCl and the resulting mixture was stirred and protected from light, for 96 hours at room temperature. The product was collected by filtration, washed well with water, and dried over P_2O_5 yielding 410 mg (82%) of **11** as a cream-colored solid. $R_f = 0.78$ (EtOH-CH_2Cl_2, 1:10). Mp 218°C–220°C. Lit.: Mp 226°C.[15] ^{1}H NMR (DMSO-d_6): 12.94 (br s, 1H, N-H), 8.52 (br s, 0.97H, N-H), 8.40 (s, 0.56H, 8-H), 8.31 (s, 0,03H, N-H), 8.12 (br s, 0.03H, 8-H), 8.04 (br s, 0.41H, 8-H), 7.12–7.61 (m, 5H, Ph), 5.41 (d, $^3J = 6.4$Hz, 0.06H, CH_2N), 4.82 (d, $^3J = 5.4$ Hz, 1.12H, CH_2N), 4.63 (br s, 0.82H, CH_2N). UV (H_2O), λ_{max} in nm (ε): pH 2, 237 (19200), 277 (15300); pH 7, 236 (21800), 273 (15600); pH 12, 236 (26100), 278 (13400).

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