

5-Substituted 2-amino-4,6-dihydroxypyrimidines and 2-amino-4,6-dichloropyrimidines: synthesis and inhibitory effects on immune-activated nitric oxide production

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Abstract A series of 5-substituted 2-amino-4,6-dihydroxypyrimidines were prepared by a modified condensation of the corresponding monosubstituted malonic acid diesters with guanidine in an excess of sodium ethoxide. The optimized procedure using Vilsmeier–Haack–Arnold reagent, followed by immediate deprotection of the (dimethylamino)methylene protecting groups, has been developed to convert the 2-amino-4,6-dihydroxypyrimidine analogs to novel 5-substituted 2-amino-4,6-dichloropyrimidines in high yields. Pilot screening for biological properties of the prepared compounds was done in mouse peritoneal cells using the *in vitro* nitric oxide (NO) assay. Irrespective of the substituent at the 5 position, 2-amino-4,6-dichloropyrimidines inhibited immune-activated NO production. The most effective was 5-fluoro-2-amino-4,6-dichloropyrimidine with an IC_{50} of 2 μ M (higher activity than the most potent reference compound) while the IC_{50} s of other derivatives were within the range of 9–36 μ M. The 2-amino-4,6-dihydroxypyrimidine counterparts were

devoid of any NO-inhibitory activity. The compounds had no suppressive effects on the viability of cells. The Mechanism of action remains to be elucidated.

Keywords Pyrimidine · Nitric oxide · NO · Anti-inflammatory

Introduction

This work was initially motivated by the finding that 2-amino-4,6-dichloropyrimidine inhibits replication of a broad range of viruses such as members of the Herpes, Picorna, and Pox groups (Marcialis *et al.*, 1973, 1974; La Colla *et al.*, 1975). It has been reported that maturation of viral particles was prevented as viral proteins synthesized in the presence of this compound were not assembled into new virions (Flore *et al.*, 1977; La Colla *et al.*, 1977; Marcialis *et al.*, 1979). This represents a unique and very attractive mechanism of action for new antiviral drugs, especially in combination with other antiviral agents with the aim to suppress resistance development. The pyrimidine heterocycle is an elemental structural motif of several essential natural products (Undheim and Benneche, 1996; Ralevic and Burnstock, 1998; Lagoja, 2005) and a number of synthetic drugs. It was also reported that 2,4-diamino-6-hydroxypyrimidine suppresses NO production in chicken macrophages (Sung *et al.*, 1994).

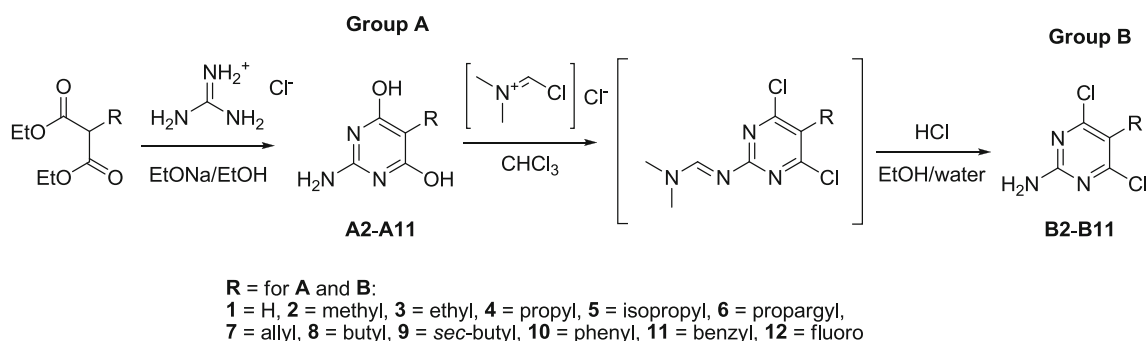
There is a large class of pharmacologically important pyrimidine derivatives that act as dihydrofolate reductase (DHFR) inhibitors (Hitchings, 1989), as well as compounds with anti-HIV (Holý, 2003; De Clercq and Holý, 2005), anti-adenovirus (Naesens *et al.*, 2005), and anti-HBV activities (Ying *et al.*, 2005), inhibitors of tetrahydrobiopterin synthesis (Bogdan *et al.*, 1995; Kolinsky and

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Scheme 1 General synthesis of 5-substituted 2-amino-4,6-dihydroxypyrimidines (**a**) and 2-amino-4,6-dichloropyrimidines (**b**). For the source of **A1**, **B1**, **A12**, and **B12** see experimental

Gross, 2004), regulators of pain sensitivity and persistence (Tegeger *et al.*, 2006), antidepressants (Arvanitis *et al.*, 1999), compounds which suppress accumulation of cytokine-induced NF- κ B (Ikemoto *et al.*, 2008), inhibitors of EGFR and Her-2 tyrosine kinases (Suzuki *et al.*, 2012) and cyclin-dependent kinases as potential drug candidates for cancer therapy (Beattie *et al.*, 2003; Breault *et al.*, 2003). Substitution at position 5 of the pyrimidine moiety has been used in the past to improve the biological property of several pharmacologically interesting pyrimidine derivatives with anti-HIV (Hocková *et al.*, 2003, 2004) or anti-influenza activity (Jansa *et al.*, 2012), with inhibitory activity against human thymidine phosphorylase (Nencka *et al.*, 2007) or with antioxidative activity (Procházková *et al.*, 2012a, b).

The aim of the present study was the preparation of 5-substituted 2-amino-4,6-dihydroxypyrimidines and 2-amino-4,6-dichloropyrimidines, and the pilot screening of their intrinsic biological potential. Substituents at position 5 of the pyrimidine ring were designed to probe this position with a standard homologous series of alkyl substituents to study steric requirements in this position (e.g., length, branching, saturation, and desaturation). Possible antiviral activity of compounds and their influence on the immune-activated high output nitric oxide (NO) production were analyzed. The NO assay has been employed for its capacity to indicate both positive and negative interferences of compounds with cell immune responses including secretion of cytokines and cytotoxicity (Kmoníčková *et al.*, 2007; Harmatha *et al.*, 2013).

Chemistry

At first, 2-amino-4,6-dihydroxypyrimidines **A2–A11** (Scheme 1) were prepared by the condensation of the properly substituted malonic acid diesters with guanidine under basic conditions. Despite the fact that this reaction is well-known in the literature (Rembold and Schramm,

1963; Baraldi *et al.*, 2003; Patel *et al.*, 2007; Rostom *et al.*, 2009), optimization of the reaction conditions in our case had a crucial impact on the isolation, yield, and purity of the products especially in multi-gram scales (for details see experimental). The following is our key findings: methanol or ethanol is required as the solvent to achieve full conversion and the clean reaction profile (alcohols are able to at least partially dissolve the product which otherwise precipitates in quantities that quickly precludes stirring); addition of water after the reaction (which dissolves the sodium salt of the product) and subsequent neutralization by acetic acid is required to keep excess of guanidine in solution while the product quantitatively precipitates from the neutralized solution.

The second step in the synthesis of the targeted 5-substituted 2-amino-4,6-dichloropyrimidines **B2–B11** (Scheme 1) consists of the transformation of the 4,6-dihydroxypyrimidine moiety into the 4,6-dichloropyrimidine residue. For such reactions, chlorides of mineral acids such as POCl₃, PCl₅, SOCl₂, or COCl₂ with diverse additives like DMF, pyridine, 2-methylpyridine, diphenylamine, or triethylamine are generally used (Altenbach *et al.*, 2008; Jang *et al.*, 2011). Nevertheless, these classical procedures turned out to be unsuitable for the preparation of the desired 5-substituted 2-amino-4,6-dichloropyrimidines **B2–B11**, owing to their complicated isolations and very low isolated yields (max. 30 %). This synthetic issue was resolved by application of a modified synthetic procedure for the preparation of 4,6-dichloro-2,5-bis{[(dimethylamino)methylene]amino}pyrimidine (Daluge *et al.*, 2000). The use of the Vilsmeier–Haack–Arnold reagent (Reichardt, 1999), followed by immediate deprotection of the (dimethylamino)methylene protecting groups, gave final compounds **B2–B11** (Scheme 1). The final step was carried out according to the method described in the literature (Daluge *et al.*, 2000), which was modified in order to obtain **B2–B11** directly from the reaction mixture by simple precipitation and filtration (for details see experimental).

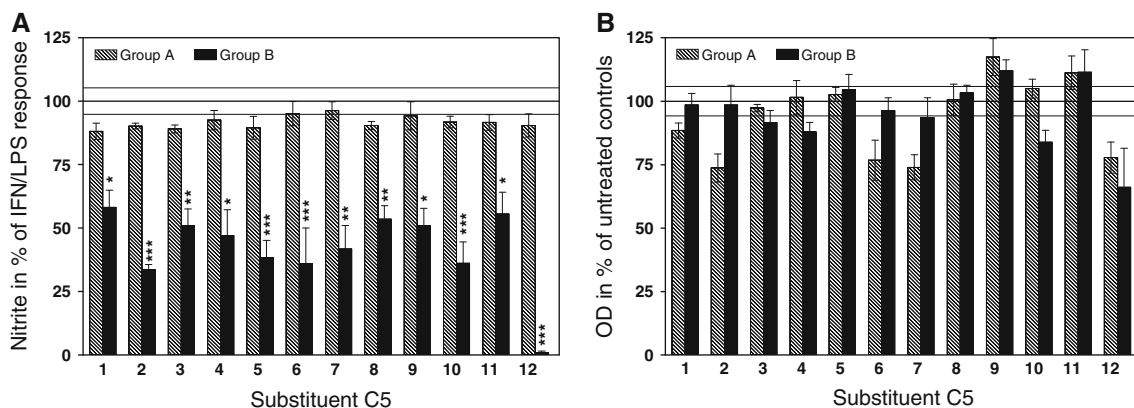


Fig. 1 Effects of 5-substituted 2-amino-4,6-dihydroxypyrimidines **A1–A12** and 2-amino-4,6-dichloropyrimidines **B1–B12** on the production of NO by mouse peritoneal cells (**a**) and their viability (**b**). The compounds were applied at a concentration of 50 μM . The NO

data are means obtained from at least four experiments. The viability data were averaged from two to four experiments. All columns are mean \pm SEM. Dotted lines denote 95 % confidential intervals of control values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Biological properties

Antiviral

None of the compounds **A1–A12** or **B1–B12** showed any antiviral activity ($\text{EC}_{50} > 100 \mu\text{g/mL}$) toward the following viruses: HIV-1, HIV-2 in CEM cells, HCMV, HSV-1, HSV-2, vaccinia virus and vesicular stomatitis virus (VSV) in HeLa cells, Feline herpes virus and Feline corona virus (FIPV) in CRFK cells, VSV, Coxsackie virus B4 and respiratory syncytial virus (RSV) in HeLa cells, Parainfluenza 3-virus, Reovirus-1, Coxsackie B4 virus and Punta Toro virus in MDCK cells. The parent 2-amino-4,6-dichloropyrimidine (**B1**) was also tested and no antiviral activity was observed ($\text{EC}_{50} > 100 \mu\text{g/mL}$). It is important to mention that antiviral activities described in the literature for this compound required relatively high concentrations in the range of the 100 $\mu\text{g/mL}$ (Marcialis *et al.*, 1973, 1974, La Colla *et al.*, 1975).

NO production

The *in vitro* stimulation of mouse peritoneal cells with LPS plus IFN- γ substantially enhanced NO biosynthesis. The concentration of nitrites varied from $53.30 \pm 0.90 \mu\text{M}$ to $74.00 \pm 3.30 \mu\text{M}$ (mean \pm SEM) in various experiments. The spontaneous production of NO by control cells was negligible, ranging from $0.80 \pm 0.30 \mu\text{M}$ to $1.70 \pm 1.60 \mu\text{M}$ (mean \pm SEM) in individual experiments.

None of the 5-substituted 2-amino-4,6-dihydroxypyrimidines **A1–A12** at 50 μM concentrations significantly influenced ($F_{12, 397} = 0.85$, $p = 0.61$) the IFN/LPS-activated NO biosynthesis (Fig. 1a). On average, the formation of nitrites dropped to 91.6 % of control values. The

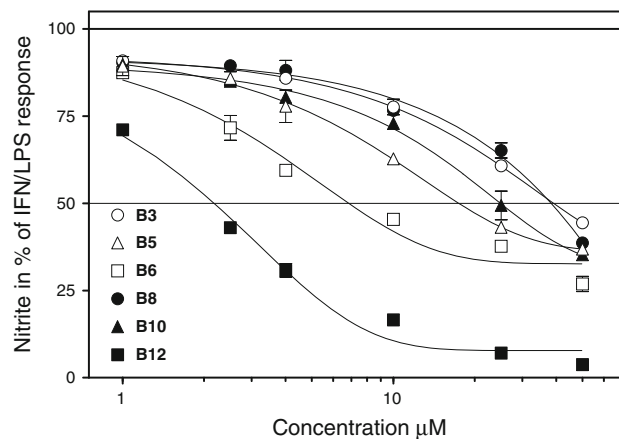


Fig. 2 Dose-dependent inhibitory effects of 5-substituted 2-amino-4,6-dichloropyrimidines **B1–B12** on *in vitro* NO production. The points are mean \pm SEM. The data represent one of two identical experiments. The concentration of nitrites following the LPS/IFN stimulus (*i.e.* 100 % response) was $67.90 \pm 1.50 \mu\text{M}$. It was $1.80 \pm 0.30 \mu\text{M}$ in untreated controls

compounds remained ineffective even at fourfold higher (*i.e.*, 200 μM) concentrations (data not shown).

In contrast, all 5-substituted 2-amino-4,6-dichloropyrimidines **B1–B12**, applied at 50 μM concentrations, suppressed NO production by at least 55 % (Fig. 1a). The effects were highly statistically significant ($F_{12, 667} = 6.04$, $p < 0.0001$). The most effective was 5-fluoro-2-amino-4,6-dichloropyrimidine (**B12**).

The suppression of NO production by 5-substituted 2-amino-4,6-dichloropyrimidines **B1–B12** was strictly dose-dependent (Fig. 2). The effects of **B6** and **B12** were already apparent at concentrations of 2.5 μM . The IC_{50} s estimates (Table 1) ranged between 2 μM and 36 μM (**B12** and **B8**, respectively).

Table 1 The NO-inhibitory IC_{50} s

	IC_{50} (μ M)	95 % Limits of confidence
5-Substituted 2-amino-4,6-dichloropyrimidines		
B3	35.7	26.78–47.59
B5	19.64	15.76–24.48
B6	9	6.26–12.93
B8	36.23	22.83–57.49
B10	24.23	18.18–32.31
B12	1.99	1.81–2.19
Reference NO-inhibitors		
Aminoguanidine	77.83	52.82–114.70
L-NMMA	35.13	20.70–59.63
L-NIL	8.14	5.83–11.36
1,400 W	3.87	2.94–5.11

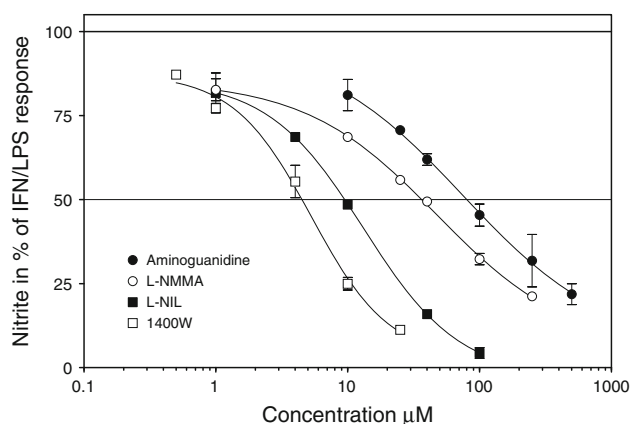


Fig. 3 Dose-dependent inhibitory effects of standard NO-inhibitors on in vitro NO production. The points are mean \pm SEM. The data represent one of two identical experiments. The concentration of nitrites following the LPS/IFN stimulus (*i.e.* 100 % response) was $64.10 \pm 1.50 \mu\text{M}$. It was $0.30 \pm 0.20 \mu\text{M}$ in untreated controls. L-NMMA, N^G -monomethyl-L-arginine; L-NIL, L - N^G -(1-iminoethyl)lysine; 1,400 W, N -(3-aminomethyl)-benzylacetamide

The NO-inhibitory effects of tested pyrimidines were compared to the effects of recognized NO-inhibitors (Fig. 3). The most effective proved to be 1,400 W; the least effective was aminoguanidine. The corresponding IC_{50} s are shown in Table 1. They are in good agreement with activities reported previously (Stenger *et al.*, 1995; Xu and Krukoff, 2005).

Cell viability

Applied at 50 μM concentrations, the compounds had no statistically significant influence on the viability of cells (Fig. 1b). No difference between the two groups of pyrimidine derivatives was observed ($F_{1, 35} = 0.25$, $p = 0.62$).

Conclusions

An optimized synthetic procedure for the preparation of 5-substituted 2-amino-4,6-dichloropyrimidines **B1–B12** starting from the monosubstituted malonic acid diesters has been developed. Final compounds **B1–B12** inhibit NO production in mouse peritoneal cells. Compounds **B5**, **B6**, **B10**, and **B12** are more potent than the most frequently used inhibitor of the NO production (N^G -monomethyl-L-arginine). The most effective was compound **B12** bearing the fluorine atom at position 5 of the pyrimidine ring, which nearly completely suppressed the production of NO and which exhibited higher activity than the most potent reference compound (1,400 W).

This interesting discovery of the inhibitory effect of the 5-substituted 2-amino-4,6-dichloropyrimidines on NO production in vitro represents an initial hit in our development of potent compounds with anti-inflammatory activity (patent pending). Compounds of the next generations and further in vitro and in vivo data will be published elsewhere.

Experimental

General

Unless stated otherwise, solvents were evaporated at 40 $^{\circ}\text{C}$ /2 kPa and compounds were dried at 13 Pa. Analytical TLC was performed on silica gel 60 F₂₅₄ plates (Merck KGaA, Darmstadt, Germany). The NMR spectra were measured on an FT NMR spectrometer (Bruker Avance II 500) in DMSO- d_6 (^1H at 500 MHz and ^{13}C at 125.7 MHz), referenced to the residual solvent signal, chemical shift are expressed in parts per million, ppm, and interaction constants J in Hz. GC/MS analyses were measured using a 6,890 N gas chromatograph (Agilent, Santa Clara, CA, USA) attached to a quadrupole mass detector. A HP-5 ms capillary (30 m \times 0.25 mm; 0.25 μm ; Agilent) was used for the analyses. The carrier gas was helium with a flow rate of 1 mL/min. The EI mass spectra were measured on a GCT Premier (Waters) OA-TOF GC mass spectrometer. The elemental composition of the prepared compounds was determined using a PE 2400 Series II CHNS/O Elemental Analyzer (Perkin Elmer, USA, 1999). Melting points were determined on a Stuart SMP3 Melting Point Apparatus and are uncorrected. Compounds **A12** and **B12** were prepared according to the literature (Schostarez, 1992). Compounds **A1** and **B1** were purchased from Sigma-Aldrich.

General procedure for the preparation of 5-substituted 2-amino-4,6-dihydroxypyrimidines **A2–A11**

Metallic sodium (12.9 g, 0.56 mol) was dissolved in absolute ethanol (300 mL) under argon while being intensively

stirred with a mechanical stirrer. The reaction flask was equipped with a reflux condenser with a chlorocalcium tube. After all the sodium was dissolved and the reaction mixture was cooled to room temperature; guanidine hydrochloride (21.02 g, 0.22 mol) was added under intensive stirring, followed by the corresponding monosubstituted malonic acid diester (0.2 mol). The reaction mixture was further intensively stirred due to the production of the solid product, which is so massive that after 2 h it already practically precludes stirring. After another 2 h, absolute ethanol (200 mL) was added and the reaction mixture was refluxed for 1 h while being stirred. Afterward, ethanol (ca 200–300 mL) was evaporated on a vacuum rotary evaporator and water (500 mL) was added to the reaction mixture. After stirring, the product (in the form of sodium salt) was almost dissolved. The obtained mixture was subsequently neutralized by dropwise addition of acetic acid, resulting in immediate and quantitative precipitation of the desired product in the form of a fine solid. This mixture was subsequently heated under reflux for 10 min and then cooled to laboratory temperature. This heating and cooling was repeated twice to get a well-filterable solid product. The solid product was filtered off, washed with water (2 × 50 mL), ethanol (2 × 50 mL), and acetone (2 × 50 mL). The product was dried under high vacuum at 60 °C for 2 days. The obtained purity of the product prepared in this manner is sufficient for the following reaction and based on analyses contains only crystalline water.

2-Amino-5-methylpyrimidine-4,6-diol (**A2**)

It was obtained as a white solid; yield: 30.86 g (91 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.70 (2H, bs, 2 × OH), 6.88 (2H, bs, NH₂), 1.57 (3H, s, H-1'); ¹³C NMR (DMSO-*d*₆): δ = 164.97 (C-4 and 6), 152.53 (C-2), 84.06 (C-5), 8.11 (C-1'); Anal. Calcd. for C₅H₇N₃O₂ + 1.6 H₂O: C, 35.34; H, 6.05; N, 24.72. Found: C, 35.57; H, 6.15; N, 24.59.

2-Amino-5-ethylpyrimidine-4,6-diol (**A3**)

It was obtained as a white solid; yield: 32.54 g (88 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.30 (2H, bs, 2 × OH), 6.30 (2H, bs, NH₂), 2.14 (2H, q, *J*(1',2') = 7.3, H-1'), 0.88 (3H, t, *J*(2',1') = 7.3, H-2'); ¹³C NMR (DMSO-*d*₆): δ = 164.47 (C-4 and 6), 152.54 (C-2), 91.88 (C-5), 15.62 (C-1'), 13.89 (C-2'); Anal. Calcd. for C₆H₉N₃O₂ + 1.7 H₂O: C, 38.79; H, 6.73; N, 22.62. Found: C, 38.83; H, 7.90; N, 22.41.

2-Amino-5-propylpyrimidine-4,6-diol (**A4**)

It was obtained as a white solid; yield: 33.86 g (94 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.30 (2H, bs, 2 ×

OH), 6.31 (2H, bs, NH₂), 2.10 (2H, t, *J*(1',2') = 7.5, H-1'), 1.32 (2H, m, H-2'), 0.80 (3H, t, *J*(3,2') = 7.4, H-3'); ¹³C NMR (DMSO-*d*₆): δ = 164.75 (C-4 and 6), 152.57 (C-2), 90.20 (C-5), 22.44 (C-1'), 21.89 (C-2'), 14.17 (C-3'); Anal. Calcd. for C₇H₁₁N₃O₂ + 0.6 H₂O: C, 46.71; H, 6.83; N, 23.35. Found: C, 46.80; H, 6.79; N, 23.32.

2-Amino-5-isopropylpyrimidine-4,6-diol (**A5**)

It was obtained as a white solid; yield: 32.36 g (93 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.45 (2H, bs, 2 × OH), 6.62 (2H, bs, NH₂), 2.96 (1H, sept, *J*(CH,CH₃) = 7.1, H-CH), 1.08 (6H, d, *J*(CH₃,CH) = 7.1, 2 × CH₃); ¹³C NMR (DMSO-*d*₆): δ = 164.19 (C-4 and 6), 152.40 (C-2), 94.80 (C-5), 22.94 (CH), 20.96 (CH₃); Anal. Calcd. for C₇H₁₁N₃O₂ + 0.3 H₂O: C, 48.16; H, 6.70; N, 24.07. Found: C, 48.11; H, 7.62; N, 23.98.

2-Amino-5-(prop-2-yn-1-yl)pyrimidine-4,6-diol (**A6**)

It was obtained as a white solid; yield: 33.47 g (96 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.55 (2H, bs, 2 × OH), 6.79 (2H, bs, NH₂), 2.95 (2H, d, *J*(1,3') = 2.6, H-1'), 2.43 (1H, t, *J*(3',1') = 2.6, H-3'); ¹³C NMR (DMSO-*d*₆): δ = 163.69 (C-4 and 6), 152.69 (C-2), 89.05 (C-2'), 84.97 (C-5), 68.02 (C-3'), 12.06 (C-1'); Anal. Calcd. for C₇H₇N₃O₂ + 0.5 H₂O: C, 48.28; H, 4.63; N, 24.13. Found: C, 48.09; H, 4.33; N, 24.23.

5-Allyl-2-aminopyrimidine-4,6-diol (**A7**)

It was obtained as a white solid; yield: 38.57 g (95 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.35 (2H, bs, 2 × OH), 6.40 (2H, bs, NH₂), 5.73 (1H, ddt, *J*(2',1') = 6.1, *J*(2',3'_{cis}) = 10.0, *J*(2',3'_{trans}) = 17.2, H-2'), 4.87 (1H, ddt, *J*(3'_{trans},1') = 1.6, *J*(gem) = 2.3, *J*(3'_{trans},2') = 17.2, H-3'_{trans}), 4.79 (1H, ddt, *J*(3'_{cis},1') = 1.6, *J*(gem) = 2.3, *J*(3'_{cis},2') = 10.0, H-3'_{cis}), 2.85 (2H, dt, *J*(1',3') = 1.6, *J*(1',2') = 6.1, H-1'); ¹³C NMR (DMSO-*d*₆): δ = 164.36 (C-4 and 6), 152.71 (C-2), 137.73 (C-2'), 113.32 (C-3'), 87.73 (C-5), 26.67 (C-1'); Anal. Calcd. for C₇H₉N₃O₂ + 2 H₂O: C, 41.38; H, 6.45; N, 20.68. Found: C, 41.44; H, 6.17; N, 20.47.

2-Amino-5-butylpyrimidine-4,6-diol (**A8**)

It was obtained as a white solid; yield: 39.61 g (97 %); m.p. >250 °C; ¹H NMR (DMSO-*d*₆): δ = 10.30 (2H, bs, 2 × OH), 6.32 (2H, bs, NH₂), 2.12 (2H, t, *J*(1',2') = 7.1, H-1'), 1.28 and 1.23 (2 × 2H, 2 × m, H-2' and H-3'), 0.85 (3H, t, *J*(4',3') = 7.2, H-4'); ¹³C NMR (DMSO-*d*₆): δ = 164.67 (C-4 and 6), 152.54 (C-2), 90.36 (C-5), 31.04 (C-1'), 22.32 (C-2'), 21.98 (C-3'), 14.22 (C-4'); Anal. Calcd. for

$C_8H_{13}N_3O_2 + 1.2 H_2O$: C, 46.91; H, 7.58; N, 20.52. Found: C, 47.15; H, 7.69; N, 20.36.

2-Amino-5-(sec-butyl)pyrimidine-4,6-diol (**A9**)

It was obtained as a white solid; yield: 36.89 g (93 %); m.p. >250 °C; 1H NMR (DMSO- d_6): $\delta = 10.20$ (2H, bs, 2 × OH), 6.31 (2H, bs, NH_2), 2.70 (1H, m, H-1'), 1.65 and 1.40 (2 × 1H, 2 × m, H-2'), 1.06 (3H, d, $J(1'',1') = 7.0$, H-1''), 0.72 (3H, t, $J(3',2') = 7.4$, H-3'); ^{13}C NMR (DMSO- d_6): $\delta = 164.46$ (C-4 and 6), 152.47 (C-2), 93.61 (C-5), 30.08 (C-1'), 27.09 (C-2'), 19.00 (C-1''), 13.03 (C-3'); Anal. Calcd. for $C_8H_{13}N_3O_2 + 0.8 H_2O$: C, 48.62; H, 7.45; N, 21.26. Found: C, 48.57; H, 7.47; N, 21.18.

2-Amino-5-phenylpyrimidine-4,6-diol (**A10**)

It was obtained as a white solid; yield: 41.43 g (94 %); m.p. >250 °C; 1H NMR (DMSO- d_6): $\delta = 10.60$ (2H, bs, 2 × OH), 7.50 (2H, d, phenyl), 7.19 (2H, t, phenyl), 7.02 (1H, t, phenyl), 6.74 (2H, bs, NH_2); ^{13}C NMR (DMSO- d_6): $\delta = 162.84$ (C-4 and 6), 152.02 (C-2), 135.40, 130.26, 126.84 and 124.19 (C-phenyl), 106.11 (C-5); Anal. Calcd. for $C_{10}H_9N_3O_2 + 1 H_2O$: C, 54.29; H, 5.01; N, 19.00. Found: C, 54.17; H, 5.19; N, 18.82.

2-Amino-5-benzylpyrimidine-4,6-diol (**A11**)

It was obtained as a white solid; yield: 43.21 g (91 %); m.p. >250 °C; 1H NMR (DMSO- d_6): $\delta = 10.42$ (2H, bs, 2 × OH), 7.18 (4H, m, phenyl), 7.07 (1H, m, phenyl), 6.46 (2H, bs, NH_2), 3.44 (2H, s, CH_2); ^{13}C NMR (DMSO- d_6): $\delta = 164.47$ (C-4 and 6), 152.68 (C-2), 143.10, 128.31, 127.90 and 125.20 (C-phenyl), 94.51 (C-5), 28.12 (CH_2); Anal. Calcd. for $C_{11}H_{11}N_3O_2 + 1.1 H_2O$: C, 55.74; H, 5.61; N, 17.73. Found: C, 55.71; H, 5.54; N, 17.60.

General procedure for the preparation of 5-substituted 2-amino-4,6-dichloropyrimidines **B2–B11**

Prior to the reaction, the starting 5-substituted 2-amino-4,6-dihydroxypyrimidine **A2–A11** was dried in a vacuum drier at 80 °C and under 0.1 mbar for 1 day, because crystalline water increases the amount of the Vilsmeier–Haack–Arnold reagent required for full conversion. Subsequently, 5-substituted 2-amino-4,6-dihydroxypyrimidine (10 mmol) was suspended under inert atmosphere in a 2 M solution of the Vilsmeier–Haack–Arnold reagent (80 mmol, 40 mL) in chloroform. The reaction mixture was subsequently heated at reflux for 4 h, during which the starting material was completely dissolved. The reaction mixture was cooled to the room temperature, poured onto ice and rapidly neutralized with a saturated aqueous $NaHCO_3$ solution.

The obtained mixture was quickly transferred into a separatory funnel and immediately extracted with chloroform (3 × 20 mL). The organic fractions were combined together, dried over $MgSO_4$, filtered through a 0.5 cm layer of silica gel and concentrated down on a rotary evaporator. This crude residue of 5-substituted 4,6-dichloro-2-[[dimethylamino)methylene]amino}pyrimidine was dissolved in the mixture of 99 % ethanol (20 mL) and 37 % aqueous HCl (2 mL). The reaction mixture was heated at 50 °C for 2 h, during which a crystalline product began to precipitate directly from the reaction mixture. After that, water (30 mL) was added and the reaction mixture was intensively stirred for 10 min. The precipitated product was filtered off and washed with a water/ethanol mixture (1/1, 2 × 10 mL), 5 % aqueous solution of $NaHCO_3$ (10 mL) and a water/ethanol mixture (1/1, 10 mL). The product was subsequently recrystallized from aqueous ethanol, filtered off, washed with a water/ethanol mixture (1/1, 10 mL) and dried under high vacuum.

4,6-Dichloro-5-methylpyrimidin-2-amine (**B2**)

It was obtained as a white solid; yield: 1.26 g (71 %); m.p. 189–190 °C; 1H NMR (DMSO- d_6): $\delta = 7.26$ (2H, bs, NH_2), 2.17 (3H, s, H-1'); ^{13}C NMR (DMSO- d_6): $\delta = 161.01$ (C-4 and 6), 160.78 (C-2), 113.60 (C-5), 14.93 (C-1'); Anal. Calcd. for $C_5H_5Cl_2N_3$: C, 33.73; H, 2.83; Cl, 39.83; N, 23.60. Found: C, 33.53; H, 2.78, Cl, 40.02; N, 23.42; MS (EI), m/z (%): 177 and 179 [M^+] (100); MS (ESI +), m/z (%): 178 and 180 [$M+H^+$] (100).

4,6-Dichloro-5-ethylpyrimidin-2-amine (**B3**)

It was obtained as a white solid; yield: 1.58 g (82 %); m.p. 183–185 °C; 1H NMR (DMSO- d_6): $\delta = 7.32$ (2H, bs, NH_2), 2.61 (2H, q, $J(1',2') = 7.4$, H-1'), 1.07 (3H, t, $J(2',1') = 7.4$, H-2'); ^{13}C NMR (DMSO- d_6): $\delta = 160.83$ (C-2), 160.76 (C-4 and 6), 118.90 (C-5), 22.41 (C-1'), 12.92 (C-2'); Anal. Calcd. for $C_6H_7Cl_2N_3$: C, 37.52; H, 3.67; Cl, 36.92; N, 21.88. Found: C, 37.59; H, 3.73; Cl, 36.76; N, 21.77; MS (EI), m/z (%): 191 and 193 [M^+] (100).

4,6-Dichloro-5-propylpyrimidin-2-amine (**B4**)

It was obtained as a white solid; yield: 1.60 g (78 %); m.p. 182–183 °C; 1H NMR (DMSO- d_6): $\delta = 7.52$ (2H, bs, NH_2), 2.69 (2H, t, $J(1',2') = 7.6$, H-1'), 1.52 (2H, m, H-2'), 0.91 (3H, t, $J(3',2') = 7.2$, H-3'); ^{13}C NMR (DMSO- d_6): $\delta = 160.78$ (C-2), 160.69 (C-4 and 6), 119.20 (C-5), 31.12 (C-1'), 20.86 (C-2'), 13.75 (C-3'); Anal. Calcd. for $C_7H_9Cl_2N_3$: C, 40.80; H, 4.40; Cl, 34.41; N, 20.39. Found: C, 40.79; H, 4.32; Cl, 34.15; N, 20.19; MS (EI), m/z (%): 205 and 207 [M^+] (100).

4,6-Dichloro-5-isopropylpyrimidin-2-amine (**B5**)

It was obtained as a white solid; yield: 1.43 g (69 %); m.p. 175–176 °C; ^1H NMR (DMSO- d_6): δ = 7.31 (2H, bs, NH₂), 3.46 (1H, sept, $J(\text{CH}, \text{CH}_3) = 7.2$, CH), 1.28 (6H, d, $J(\text{CH}_3, \text{CH}) = 7.2$, $2 \times \text{CH}_3$); ^{13}C NMR (DMSO- d_6): δ = 160.62 (C-2), 160.32 (C-4 and 6), 121.65 (C-5), 28.57 (CH), 19.82 (CH₃); Anal. Calcd. for C₇H₉Cl₂N₃: C, 40.80; H, 4.40; Cl, 34.41; N, 20.39. Found: C, 40.57; H, 4.54; Cl, 34.69; N, 20.67; MS (EI), m/z (%): 205 and 207 [M^+] (100).

4,6-Dichloro-5-(prop-2-yn-1-yl)pyrimidin-2-amine (**B6**)

It was obtained as a white solid; yield: 1.63 g (81 %); m.p. 159–161 °C; ^1H NMR (DMSO- d_6): δ = 7.50 (2H, bs, NH₂), 3.52 (2H, d, $J(1', 3') = 2.7$, H-1'), 2.96 (1H, t, $J(3', 1') = 2.7$, H-3'); ^{13}C NMR (DMSO- d_6): δ = 161.20 (C-2), 160.84 (C-4 and 6), 113.30 (C-5), 79.86 (C-2'), 71.96 (C-3'), 19.03 (C-1'); Anal. Calcd. for C₇H₅Cl₂N₃: C, 41.61; H, 2.49; Cl, 35.09; N, 20.80. Found: C, 41.41; H, 2.48; Cl, 34.96; N, 20.55; MS (EI), m/z (%): 201 and 203 [M^+] (100).

5-Allyl-4,6-dichloropyrimidin-2-amine (**B7**)

It was obtained as a white solid; yield: 1.50 g (74 %); m.p. 175–177 °C; ^1H NMR (DMSO- d_6): δ = 7.40 (2H, bs, NH₂), 5.83 (1H, ddt, $J(2', 1') = 5.8$, $J(2', 3'_{\text{cis}}) = 10.1$, $J(2', 3'_{\text{trans}}) = 17.1$, H-2'), 5.06 (1H, dq, $J(3'_{\text{cis}}, 1') = J(\text{gem}) = 1.6$, $J(3'_{\text{cis}}, 2') = 10.1$, H-3'_{\text{cis}}), 4.96 (1H, dq, $J(3'_{\text{trans}}, 1') = J(\text{gem}) = 1.7$, $J(3'_{\text{trans}}, 2') = 17.1$, H-3'_{\text{trans}}), 3.36 (2H, dt, $J(1', 3') = 1.7$, $J(1', 2') = 5.8$, H-1'); ^{13}C NMR (DMSO- d_6): δ = 161.33 (C-4 and 6), 161.08 (C-2), 133.57 (C-2'), 116.28 (C-3'), 115.01 (C-5), 32.70 (C-1'); Anal. Calcd. for C₇H₇Cl₂N₃: C, 41.20; H, 3.46; Cl, 34.75; N, 20.59. Found: C, 41.12; H, 3.37; Cl, 34.54; N, 20.56; MS (EI), m/z (%): 203 and 205 [M^+] (100).

5-Butyl-4,6-dichloropyrimidin-2-amine (**B8**)

It was obtained as a white solid; yield: 1.92 g (87 %); m.p. 169–170 °C; ^1H NMR (DMSO- d_6): δ = 7.29 (2H, bs, NH₂), 2.59 (2H, m, CH₂), 1.45 (2H, m, CH₂), 1.33 (2H, m, CH₂), 0.91 (3H, t, $J(4', 3') = 7.3$, H-4'); ^{13}C NMR (DMSO- d_6): δ = 160.78 (C-2), 160.76 (C-4 and 6), 117.71 (C-5), 30.37, 28.46 and 22.04 (C-1', 2' and 3'), 13.81 (C-4'); Anal. Calcd. for C₈H₁₁Cl₂N₃: C, 43.66; H, 5.04; Cl, 32.22; N, 19.09. Found: C, 43.70; H, 4.93; Cl, 32.24; N, 18.87; GC/MS-EI (R_T 16.03 min), m/z (%): 219 and 221 [M^+] (18), 176 and 178 [M^+ -Pr] (100), min. 99.5 % purity.

5-(Sec-butyl)-4,6-dichloropyrimidin-2-amine (**B9**)

It was obtained as a white solid; yield: 1.66 g (75 %); m.p. 159–160 °C; ^1H NMR (DMSO- d_6): δ = 7.33 (2H, s, NH₂), 3.23 (1H, m, H-1'), 1.83 and 1.64 ($2 \times$ 1H, $2 \times$ m, H-2'), 1.25 (3H, d, $J(1'', 1') = 6.2$, H-1''), 0.77 (3H, t, $J(3', 2') = 7.4$, H-3'); ^{13}C NMR (DMSO- d_6): δ = 163.64 (C-2), C-4 and C-6 not found, 120.12 (C-5), 35.72 (C-1'), 26.67 (C-2'), 18.13 (C-1''), 12.53 (C-3'); Anal. Calcd. for C₈H₁₁Cl₂N₃: C, 43.66; H, 5.04; Cl, 32.22; N, 19.09. Found: C, 43.63; H, 4.82; Cl, 32.17; N, 18.86; MS (EI), m/z (%): 219 and 221 [M^+] (100).

4,6-Dichloro-5-phenylpyrimidin-2-amine (**B10**)

It was obtained as a white solid; yield: 1.71 g (71 %); m.p. 193–195 °C; ^1H NMR (DMSO- d_6): δ = 7.60 (2H, bs, NH₂), 7.44 (2H, t, phenyl), 7.40 (1H, t, phenyl), 7.30 (2H, d, phenyl); ^{13}C NMR (DMSO- d_6): δ = 161.46 (C-2), 160.26 (C-4 and 6), 134.24, 130.47, 128.55 and 128.46 (phenyl), 119.71 (C-5); Anal. Calcd. for C₁₀H₇Cl₂N₃: C, 50.03; H, 2.94; Cl, 29.53; N, 17.50. Found: C, 49.86; H, 2.82; Cl, 38.44; N, 17.23; MS (EI), m/z (%): 239 and 241 [M^+] (100).

5-Benzyl-4,6-dichloropyrimidin-2-amine (**B11**)

It was obtained as a white solid; yield: 2.02 g (80 %); m.p. 196–197 °C; ^1H NMR (DMSO- d_6): δ = 7.46 (2H, bs, NH₂), 7.29 (2H, t, phenyl), 7.20 (1H, t, phenyl), 7.15 (2H, d, phenyl), 4.01 (2H, s, CH₂); ^{13}C NMR (DMSO- d_6): δ = 161.75 (C-4 and 6), 161.15 (C-2), 138.16, 128.72, 127.92 and 126.56 (C-phenyl), 116.29 (C-5), 34.15 (CH₂); Anal. Calcd. for C₁₁H₉Cl₂N₃: C, 51.99; H, 3.57; Cl, 27.90; N, 16.54. Found: C, 51.92; H, 3.66; Cl, 28.02; N, 16.68; MS (EI), m/z (%): 253 and 255 [M^+] (100).

Preparation of the compound solutions for biological assays

The 200 mM stock solutions of compounds were prepared in DMSO. They were further diluted to working concentrations in complete RPMI-1640 culture medium (described below). In order to exclude any possible interference of DMSO with NO production and viability of cells, appropriately diluted DMSO was included in all experiments. It was found ineffective in the assays (data not shown). Standard inhibitors of NO production, i.e., *N*^G-monomethyl-L-arginine (L-NMMA), aminoguanidine (AG), L-*N*⁶-(1-iminoethyl)lysine (L-NIL), and *N*-(3-aminomethyl)benzylacetamide (1,400 W) were bought from Sigma-Aldrich. Stock solutions (5 mM) were prepared in pyrogenic distilled water.

Animals; isolation and cultivation of cells

Female mice of the inbred strain C57BL/6, 8- to 10-week-old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of ten, and maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set on 06–18 h, temperature at 22 °C.

Animals, killed by cervical dislocation, were intraperitoneally injected with 8 mL of sterile saline. Pooled peritoneal cells collected from mice ($n = 4$ –10 in individual experiments) were washed in sterile saline, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar). The final amount of cells was 2×10^6 cells/mL. The cultures were maintained at 37 °C, 5 % CO₂ in humidified Heraeus incubator.

Complete RPMI-1640 culture medium (Sigma-Aldrich) contained 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 µg/mL gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma).

All protocols were approved by the institutional ethics committee.

Nitric oxide assay

The cells were cultured 24 h in presence of the test compounds. They were applied either alone or concomitantly with the NO-enhancing stimulus provided by combination of murine recombinant interferon- γ (IFN- γ , 0.5 ng/mL; R&D Systems, Minneapolis, MN) and lipopolysaccharide (LPS from *E. coli* 055: B5, 0.1 ng/mL; Sigma). All variants were run in duplicate.

The concentration of nitrites in supernatants of cells was taken as a measure of NO production (Marletta *et al.*, 1988). It was detected in individual, cell-free samples (50 µL) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1 % sulphanilamide/0.1 % naphthylendiamine/2.5 % H₃PO₄). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to µM nitrite.

Cell viability assay

Viability of cells was determined using a colorimetric assay based on cleavage of the tetrazolium salt WST-1 (Roche Diagnostics, Mannheim, Germany) by mitochondrial dehydrogenases in viable cells. The cells were cultured quadruplicate, as described above. After the 24 h culture, the WST-1 was added and the cells were kept in the Heraeus incubator at 37 °C for additional 3 h. Optical density at 450/690 nm was evaluated.

Statistical evaluation

Analysis of variance (ANOVA) with subsequent Bonferroni's multiple comparison test and graphical presentation of data was done using the Prism program (GraphPad Software, San Diego, CA). Several experiments were performed. The concentration of nitrites as well as optical density, the measure of viability of cells, varied among experiments. In order to amalgamate the data from various experiments, they were expressed as a percentage of control values.

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