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Synthesis and immunobiological activity of base substituted 2-amino-3-(purin-9-yl)propanoic acid derivatives

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Abstract—2-Amino-3-(purin-9-yl)propanoic acids substituted at position 6 of the purine base moiety by dimethylamino, cyclopropylamino, pyrrolidin-1-yl, hydroxy, and sulfanyl group as well as their 2-aminopurine analogues were prepared from corresponding 9-(2,2-diethoxyethyl)purines and 2-aminopurines, respectively, by the Strecker synthesis. 2-Aminopropanoic acid derivatives were tested for their immunostimulatory and immunomodulatory potency. Some of these compounds significantly enhanced secretion of chemokines RANTES and MIP-1α, the most potent was 2-amino-6-sulfanylpurine derivative. Most of these compounds also augmented NO biosynthesis triggered primarily by IFN-γ.

1. Introduction

Acyclic analogues of nucleosides are intensively studied in the context of cancer therapy and viral diseases.[1] They are characterized by a significant resistance against chemical and biological degradation, which is due to the absence of a glycosidic bond. The flexibility of the acyclic chain allows adopting a suitable conformation for interaction with an active site of an enzyme or with a receptor.[2]

3-(Adenin-9-yl)-2-hydroxypropanoic acid, AHPA (**1**, Fig. 1), is a powerful inhibitor of *S*-adenosyl-L-homocysteine hydrolase.[3] AHPA interferes with systems that require massive SAM-mediated methylations catalyzed by methyl transferases. Thus, it manifests strong chemosterilizing effect on certain insects,[4] on differentiation of plant root cells[5] and also an antiviral effect directed against minus stranded RNA viruses and poxviruses.[6] The target for AHPA action is the capping process (methylation of 5′-end-guanine of viral mRNA). In our previous structure–biological activity studies[7] we have prepared new base-modified racemic AHPA derivatives bearing N^6-substituted amino function, C^6-hydroxy or sulfanyl function, and their 2-amino congeners as well. The aim of our study was to look more closely to the mechanism of AHPA antiviral action to find out whether inhibition of methylation is the only mechanism of its biological activity.

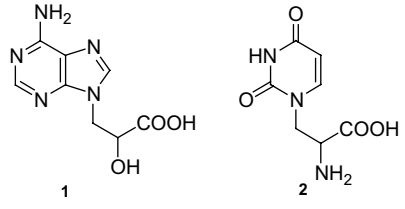

Figure 1.

In continuation of our studies we decided to replace the 2-hydroxy function at the propanoic acid by amino group in order to get access to the willardiine analogues. Willardiine, L-β-(uracil-1-yl)alanine (**2**, Fig. 1), is a naturally occurring compound found in seeds of Acacia and Mimosa.[8] Some of its analogues bearing pyrimidine and purine bases were previously described.[9] The purine analogue was prepared by Michael addition of adenine to α-chloroacrylate; the following hydrolysis and

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amination afforded the desired product.[8b,10] Such compounds were also obtained by the alkylation of purine or pyrimidine base with bromoacetaldehyde diethyl acetal affording 9- and 1-substituted product, respectively, which was hydrolyzed to the free aldehyde and converted into the α-aminopropanoic acid by the Strecker synthesis.[9b,11]

In the present paper we describe new 3-(purin-9-yl)-2-aminopropanoic acids derived from N^6-substituted adenine and 2,6-diaminopurine, the corresponding hypoxanthine and guanine derivatives as well as their sulfanyl analogues. All these compounds were tested for their antiviral and immunobiological activity. The immunobiological activities of the compounds may be considered of potential interest from the point of view of the treatment of viral infections including HIV. Among numerous other biological activities, nitric oxide (NO) and chemokines are also involved in the control of viral infections. Chemokines 'regulated upon activation, normal T cell expressed and secreted' (RANTES) and macrophage inflammatory protein-1 (MIP-1α) are natural ligands for the chemokine receptors CCR5 and CXCR4 that are also coreceptors acting during the entry of HIV-1 into the cells of the immune system, and in cooperation with CD4 receptor they ensure a productive infection.[12] Blocking the appropriate β-chemokine receptors on both macrophages and lymphocytes is presently considered as a potential therapeutic approach against HIV.[13]

NO is one of the most important effector molecules in a repertoire of non-specific immune defence mechanisms; it exhibits antiparasitic, antibacterial, and antiviral properties.[14] It is also considered to mediate inhibitory effects of IFN-γ and/or other cytokines on replication of many viruses (e.g., poxviridae, herpetoviridae, rhabdoviridae, retroviridae, hepatitis B virus,[15] cytomegalovirus,[16] Epstein–Barr virus,[17] vaccinia virus, and HIV[18]). Thus, we were interested in estimation of immunobiological activity of these simple molecules which would also be considered as analogues of phenylalanine and/or molecules with certain perspectives in peptide–nucleic acid (PNA) chemistry.

2. Results and discussion

2.1. Chemistry

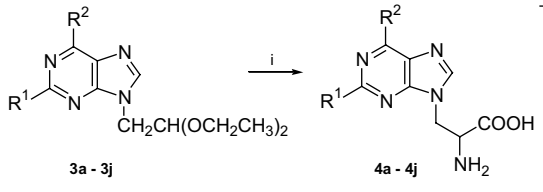
The target 2-aminopropanoic acid derivatives **4** were prepared from 9-(2,2-diethoxyethyl)purines **3** substituted at positions 2 and 6 (see Ref. 7) using the Strecker synthesis. Compounds **3a–j** were hydrolyzed to the free aldehydes with diluted HCl, the reaction mixture was treated with KCN in the presence of NH_4Cl and aqueous ammonia overnight.[19] The reaction mixture was deionized on Dowex 50 × 8 and without further purification hydrolyzed with 4 M HCl to the free acid **4**. Deionization on Dowex 50 × 8 and further purification on anion exchange resin Dowex 1 × 2 finally afforded racemic amino acids **4a–j** in moderate yields (Scheme 1). Low yields in some cases were caused by formation of side products under the conditions of the Strecker synthesis and by low solubility of amino acids that caused difficulties during purification.

2.2. Biological activity

The compounds **4a–j** were tested for their immunostimulatory and immunomodulatory potency. Specifically, their ability to stimulate secretion of chemokines, and to interfere with production of NO by macrophages was investigated.

Compound **4j** proved to be potent activator of the RANTES expression. The stimulatory effects of **4b** and **i** were less pronounced, though statistically significant (Fig. 2A). These compounds as well as compound **4e** also significantly enhanced secretion of another chemokine MIP-1α (Fig. 2B). Again, the most potent compound was **4j**. Interestingly, while **4e** was inefficient to stimulate RANTES, it was nearly as effective as its congener **4j** in producing MIP-1α. The dose–response study showed that the immunostimulatory effects are apparent at concentrations as low as 10–25 μM (Fig. 3).

None of the compounds stimulated production of NO when applied alone (data not shown). In contrast, several of them (**4b**,**d–f**,**i**,**j**) statistically significantly augmented NO biosynthesis triggered primarily by IFN-γ (Fig. 2C), the most effective being **4f** and **j**. Their NO-enhancing activity was apparent at concentrations as low as 2.5–10 μM (Fig. 4). The immunomodulatory, that is, NO augmenting effects are only partly overlapping with the potential of compounds to induce secretion of chemokines. Thus, although **4f** and **d** produce neither RANTES nor MIP-1α, they are rather prominent activators of NO biosynthesis. Production of NO is under tight control of a number of cytokines. The crucial role is played by IFN-γ that stimulates NO on its own.[20] Other cytokines like TNF-α and IL-1β, although being themselves usually ineffective, provide the major



3a - 3j 4a - 4j

3, 4	R^1	R^2	yield of 4 (%)
a	H	dimethylamino	43
b	H	cyclopropylamino	54
c	H	pyrrolidin-1-yl	30
d	H	OH	25
e	H	SH	28
f	NH_2	dimethylamino	39
g	NH_2	cyclopropylamino	30
h	NH_2	pyrrolidin-1-yl	42
i	NH_2	OH	18
j	NH_2	SH	21

Scheme 1. Reagents: (i) 1. H^+; 2. KCN, NH_4OH, NH_4Cl; 3. H^+.

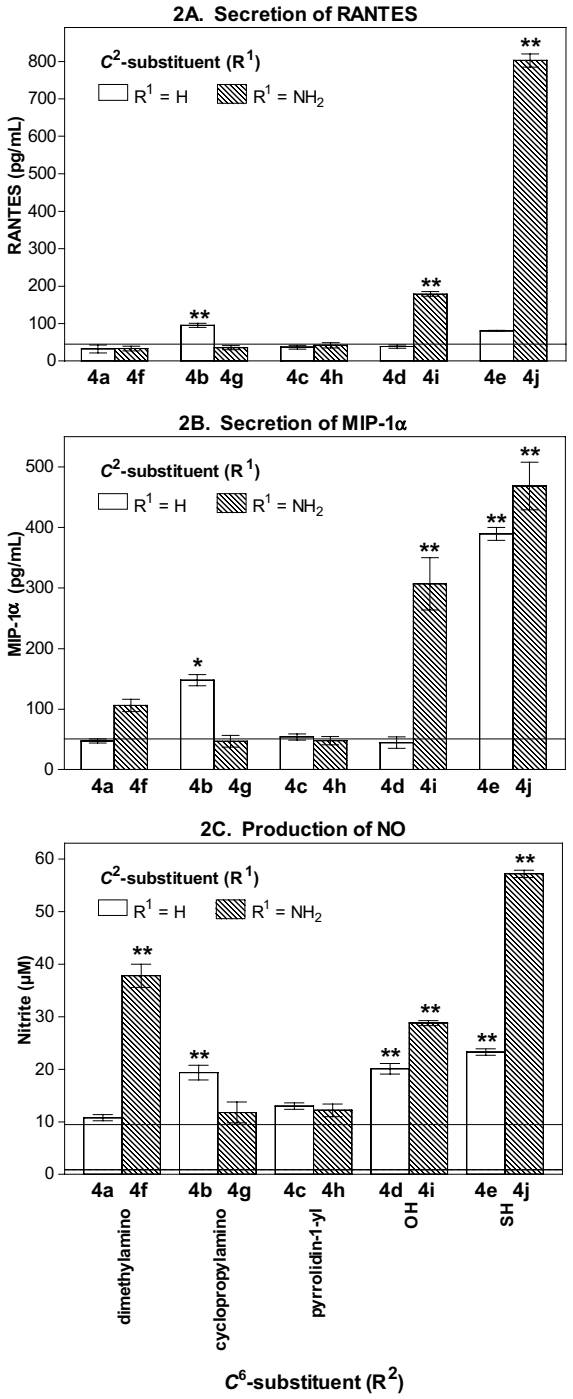


Figure 2. Stimulation of chemokine secretion (Fig. 2A and B) and modulation of NO production (Fig. 2C) by test compounds (100 μM). Their biological potential was screened in vitro using mouse resident peritoneal macrophages (2×10^6/mL). The chemokines were assayed by ELISA. Production of NO was determined in presence of compounds + IFN-γ (1000 pg/mL), given as a priming immune stimulus. Nitrite concentration was analyzed spectrophotometrically using Griess reagent. The bars are means ±SEM and are representative of at least two independent experiments. *,**—Statistically significant at $P < 0.05$, and $P < 0.01$, respectively.

synergizing signal for high-output NO release by IFN-γ-primed macrophages.[21] Thus, the incomplete correlation between the immunomodulatory and immunostimulatory effects may plausibly be explained by the lack of

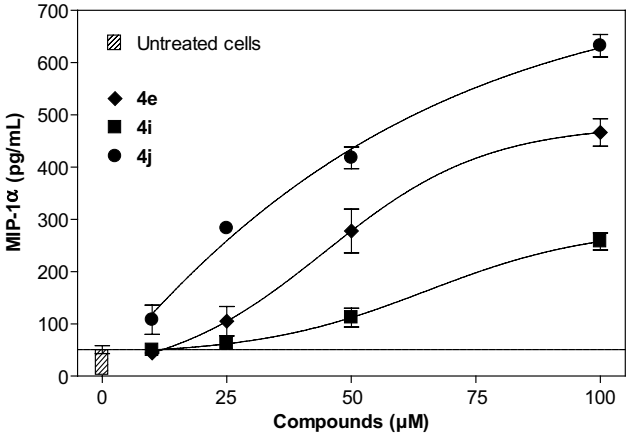


Figure 3. Secretion of MIP-1α by mouse peritoneal macrophages cultured 5 h in presence of increasing concentrations of test compounds. The chemokine was determined using the Quantikine® ELISA kit (R&D Systems, Minneapolis, USA).

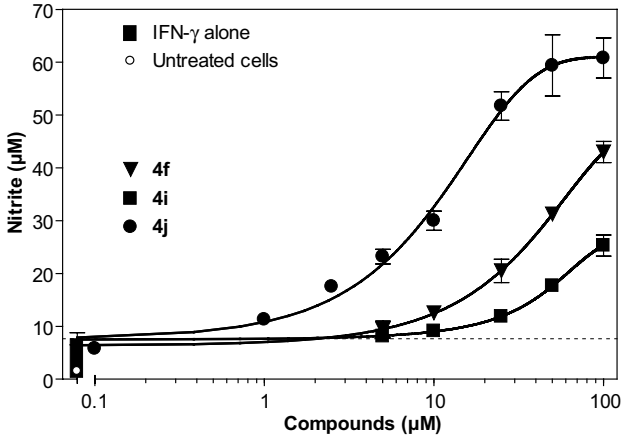


Figure 4. Production of NO by mouse peritoneal macrophages cultured 24 h in presence of increasing concentrations of test compounds, together with murine recombinant IFN-γ (1000 pg/mL). Nitrite concentration was determined spectrophotometrically using Griess reagent.

the compounds **4f** and **d** to activate chemokine expression while they may stimulate secretion of some of the NO-enhancing cytokines. This possibility remains to be ascertained.

Despite the immunobiological activity of some compounds, none of them showed any appreciable antiviral activity. Compounds **4e** and **j** were cytotoxic to human T-lymphocyte (CEM) cells, while none of them exhibited any cytostatic or cytotoxic activity in mouse leukemia L1210 cells, human T-lymphoblastoid CCRF-CEM cell line, human promyelocytic leukemia HL-60 cells and human cervix carcinoma HeLa S3 cells.

3. Experimental

Unless otherwise stated, solvents were evaporated at 40 °C/2 kPa and compounds were dried overnight at 2

kPa over P_2O_5. Melting points were determined on a Büchi Melting Point B-545 apparatus and are uncorrected. TLC was performed on plates of Kieselgel 60 F254 (Merck). Paper electrophoresis was performed on a Whatman No. 3 MM paper at 40 V/cm for 1 h in 0.05 M triethylammonium hydrogencarbonate, pH 7.5; the electrophoretical mobilities were referenced to uridine 3′-phosphate. NMR spectra were measured on an FT NMR spectrometer Varian UNITY 500 (^{1}H at 500 MHz and ^{13}C at 125.7 MHz) in dimethyl sulfoxide-d_6 or D_2O + NaOD. Chemical shifts (δ ppm) and coupling constants (J, Hz) were obtained by the first-order analysis of the spectra. Mass spectra were measured on a ZAB-EQ (VG Analytical) spectrometer using FAB (ionization by Xe, accelerating voltage 8 kV, glycerol matrix). UV spectra were measured on a UV–vis Shimadzu 1240 spectrophotometer in 0.01 M HCl, pH 2; λ_{max} and ε_{max} shown in nm and $dm^2 mmol^{-1}$, respectively.

3.1. General methods

3.1.1. Deionization of the reaction mixture. The solution of reaction products in water (20–25 mL) was applied on a column of Dowex 50 × 8 (100 mL, H^+ form), and the column was washed with water until the drop of the UV absorption (254 nm) and acid reaction of the eluate. The standard elution rate was 3 mL/min. Elution was continued with 10% ammonia, and the UV-absorbing eluate was collected and evaporated.

3.1.2. Purification by column chromatography on Dowex 1 × 2. Unless stated otherwise, 100 mL columns of Dowex 1 × 2 (acetate form) were used. The sample was dissolved in water (20–25 mL), alkalified with concentrated aqueous ammonia to pH 9–9.5, and applied on the column. Elution with water (3 mL/min) was continued until the drop of the initial UV absorption (254 nm) of the eluate. The column was then eluted with a linear gradient of acetic acid.

Compounds **3a**–**j** were prepared according to the previously described procedures.[7]

3.2. C^6-Substituted 2-amino-3-(purin-9-yl)propanoic acids (4a–e) and 2-amino-3-(2-aminopurin-9-yl)propanoic acids (4f–j)

General procedure: The corresponding C^6-substituted 9-(2,2-diethoxyethyl)purine (**3a**–**e**) or 2-amino-9-(2,2-diethoxyethyl)purine (**3f**–**j**) (4 mmol) was suspended in water; a concentrated hydrochloric acid (1.6 mL) was added and the reaction mixture was heated at 80 °C for 4–8 h. After cooling to 0 °C, the reaction mixture was neutralized with aqueous ammonia (25%) and a solution of KCN (0.65 g, 10 mmol) and NH_4Cl (0.43 g, 8 mmol) in aqueous ammonia (12 mL, 25%) was added; the mixture was stirred at rt for 12–24 h, evaporated and the residue in water was neutralized by Dowex 50 × 8 and deionized on a column of Dowex 50 × 8 (see above). The reaction products in hydrochloric acid (10 mL, 4 M) were stirred at 80 °C for 5 h, the reaction mixture was deionized on a column of Dowex 50 × 8 (see above) and purified on a column of Dowex 1 × 2 (see above). Unless otherwise stated, the column was eluted with gradient of acetic acid (2 L, 0–0.5 M). The following compounds were prepared by this procedure.

3.2.1. 2-Amino-3-[6-(dimethylamino)purin-9-yl]propanoic acid (4a). DMF (3 mL) was added to the starting reaction mixture to improve solubility. Crystallization from water afforded white product (43%), mp 250–252 °C, E_{Up} = 0.45. For $C_{10}H_{14}N_6O_2 \cdot H_2O$ (268.27) calcd: C, 44.77; H, 6.01; N, 31.33; O, 17.89. Found: C, 44.68; H, 6.06; N, 31.18. FAB MS: 251 (MH^+) (100). ^{1}H NMR (D_2O): 7.95 and 7.89 (2 × s, 2 × 1H, H-2 and H-8); 4.38 (dd, 1H, J(1′a,2′) = 5.5, J(*gem*) = 14.2, H-1′a); 4.20 (dd, 1H, J(1′b,2′) = 7.6, J(*gem*) = 14.2, H-1′b); 3.70 (dd, 1H, J(2′,1′a) = 5.5, J(2′,1′b) = 7.6, H-2″). ^{13}C NMR (D_2O): 179.06 (C=O); 153.91 (C-6); 151.55 (C-2); 149.07 (C-4); 140.30 (C-8); 118.41 (C-5); 56.08 (C-2′); 47.95 (C-1′); 38.80 (2C, N–CH_3). UV: 267.60 (16.52), 233.40 (2.78), 208.60 (16.98).

3.2.2. 2-Amino-3-[6-(cyclopropylamino)purin-9-yl]propanoic acid (4b). Crystallization from water–acetone mixture (1:1) afforded the product as a white solid (54%), mp 251 °C dec., E_{Up} = 0.59. For $C_{11}H_{14}N_6O_2$ (262.27) calcd: C, 50.38; H, 5.38; N, 32.04; O, 12.20. Found: C, 50.09; H, 5.42; N, 31.74. FAB MS: 263 (MH^+) (75). ^{1}H NMR (DMSO-d_6): 8.24 (br s, 1H, H-2); 8.01 (s, 1H, H-8); 7.92 (br s, 1H, NH); 7.90 (br, 2H, NH_2); 4.60 (dd, 1H, J(1′a,2′) = 3.5, J(*gem*) = 14.6, H-1″a); 4.37 (dd, 1H, J(1′b,2′) = 8.2, J(*gem*) = 14.6, H-1′b); 3.76 (dd, 1H, J(2′,1′a) = 3.5, J(2′,1′b) = 8.2, H-2′); 3.05 (m, 1H, N–CH); 0.71 and 0.60 (2 × m, 2 × 2H, CH_2). ^{13}C NMR (DMSO-d_6): 167.56 (C=O); 155.70 (C-6); 152.30 (C-2); 149.50 (C-4); 141.33 (C-8); 119.38 (C-5); 54.18 (C-2′); 44.89 (C-1′); 23.98 (N–CH); 6.61, 2C (CH_2). UV: 266.50 (18.67), 232.70 (3.21), 208.60 (18.63).

3.2.3. 2-Amino-3-[6-(pyrrolidin-1-yl)purin-9-yl]propanoic acid (4c). Crystallization from water afforded white solid (30%), mp 248–249 °C, E_{Up} = 0.56. For $C_{12}H_{16}N_6O_2 \cdot H_2O$ (294.31) calcd: C, 48.97; H, 6.16; N, 28.55; O, 16.31. Found: C, 48.82; H, 6.23; N, 28.31. FAB MS: 277 (MH^+) (100). ^{1}H NMR (D_2O + NaOD): 7.92 (s, 1H, H-2); 7.85 (s, 1H, H-8); 4.38 (dd, 1H, J(1′a,2′) = 5.5, J(*gem*) = 14.2, H-1′a); 4.19 (dd, 1H, J(1′b,2′) = 7,6, J(*gem*) = 14.7, H-1′b); 3.70 (dd, 1H, J(2′,1′a) = 5.5, J(2′,1′b) = 7.6, H-2′); 3.69 and 3.40 (2 × m, 2 × 2H, N–CH_2); 2.03 and 1.98 (2 × m, 2 × 2H, CH_2). ^{13}C NMR (D_2O + NaOD): 178.99 (C=O); 151.74 (C-6); 151.64 (C-2); 148.61 (C-4); 140.52 (C-8); 118.25 (C-5); 56.11 (C-2′); 48.93 and 47.90 (N–CH_2); 47.92 (C-1′); 25.57 and 23.73 (CH_2). UV: 269.10 (17.64), 232.80 (2.38), 208.70 (16.65).

3.2.4. 2-Amino-3-(hypoxantin-9-yl)propanoic acid (4d). Crystallization from water–acetone mixture afforded the product as a white solid (25%), mp 223–225 °C, E_{Up} = 0.62. For $C_8H_9N_5O_3 \cdot 3/2H_2O$ (241.24) calcd: C, 38.40; H, 4.83; N, 27.99; O, 28.77. Found: C, 38.67; H, 4.87; N, 27.70. FAB MS: 224 (MH^+) (64). ^{1}H NMR (D_2O + NaOD): 8.14 (s, 1H, H-2); 7.90 (s, 1H,

H-8); 4.40 (dd, 1H, $J(1'a,2')$ = 5.6, $J(gem)$ = 14.2, H-1′a); 4.25 (dd, 1H, $J(1'b,2')$ = 7.4, $J(gem)$ = 14.2, H-1′b); 3.74 (dd, 1H, $J(2,1'a)$ = 5.6, $J(2',1'b)$ = 7.4, H-2′). ^{13}C NMR (D_2O + NaOD): 179.39 (C=O); 167.79 (C-6); 153.70 (C-2); 150.04 (C-4); 140.67 (C-8); 123.02 (C-5); 56.22 (C-2′); 47.91 (C-1′). UV: 248.80 (7.85).

3.2.5. 2-Amino-3-(6-sulfanylpurin-9-yl)propanoic acid (4e). Crystallized from water with addition of acetic acid (pH 4), yellow crystalline product (28%), mp 222 °C dec., E_{Up} = 0.71. For $C_8H_9N_5O_2S{\cdot}H_2O$ (257.26) calcd: C, 37.35; H, 4.31; N, 27.22; O, 18.66; S, 12.46. Found: C, 37.46; H, 4.23; N, 27.37; S, 12.48. FAB MS: 240.1 (MH^+) (12). ^{1}H NMR (D_2O + NaOD): 8.31 (s, 1H, H–2); 8.08 (s, 1H, H–8); 4.46 (dd, 1H, $J(1'a,2')$ = 5.5, $J(gem)$ = 14.4, H-1′a); 4.30 (dd, 1H, $J(1'b,2')$ = 7.3, $J(gem)$ = 14.4, H-1′b); 3.75 (dd, 1H, $J(2',1'a)$ = 5.5, $J(2',1'b)$ = 7.3, H-2′). ^{13}C NMR (D_2O + NaOD): 179.31 (C-6); 177.23 (C=O); 150.92 (C-2); 146.28 (C-4); 143.01 (C-8); 135.27 (C-5); 56.18 (C-2); 48.00 (C-1′). UV: 321.40 (9.86).

3.2.6. 2-Amino-3-[2-amino-6-(dimethylamino)purin-9-yl]propanoic acid (4f). DMF (3 mL) was added to the starting reaction mixture to improve solubility. Crystallization from water–acetone (1:1) mixture gave white solid (39%), mp 243–244 °C, E_{Up} = 0.30. For $C_{10}H_{15}N_7O_2{\cdot}H_2O$ (283.29) calcd: C, 42.40; H, 6.05; N, 34.61; O, 16.94. Found: C, 42.23; H, 6.11; N, 34.31. FAB MS: 266 (MH^+) (100). ^{1}H NMR (D_2O): 7.58 (s, 1H, H-8); 4.278 (dd, 1H, $J(1'a,2')$ = 5.4, J (gem) = 14.3, H-1′a); 4.07 (dd, 1H, $J(1'b,2')$ = 7.6, $J(gem)$ = 14.3, H-1′b); 3.67 (dd, 1H, $J(2',1'a)$ = 5.4, $J(2',1'b)$ = 7.6, H-2′). ^{13}C NMR (D_2O): 179.35 (C=O); 159.29 (C-2), 154.79 (C-6); 151.55 (C-4); 138.09 (C-8); 113.32 (C-5); 56.06 (C-2′); 47.54 (C-1); 38.40, 2C (N–CH_3). UV: 293.80 (10.09), 274.30 (7.28), 256.60 (10.34).

3.2.7. 2-Amino-3-[2-amino-6-(cyclopropylamino)purin-9-yl]propanoic acid (4g). Crystallization from water–acetone (1:1) mixture gave white solid (30%), mp 252 °C, E_{Up} = 0.48. For $C_{11}H_{15}N_7O_2{\cdot}H_2O$ (295.29) calcd: C, 44.74; H, 5.80; N, 33.20; O, 16.25. Found: C, 44.94; H, 5.85; N, 32.94. FAB MS: 278 (MH^+) (100). ^{1}H NMR (D_2O): 7.69 (s, 1H, H-8); 4.33 (dd, 1H, J (1′a, 2′) = 5.4, $J(gem)$ = 14.3, H-1′a); 4.15 (dd, 1H, $J(1'b,2')$ = 7.6, $J(gem)$ = 14.3, H-1′b); 3.73 (dd, 1H, $J(2',1'a)$ = 5.4, $J(2',1'b)$ = 7.6, H-2′); 2.83 (m, 1H, N–CH); 0.92 and 0.70 (2 × m, 2 × 2H, CH_2). ^{13}C NMR (D_2O): 179.35 (C=O); 160.02 (C-2); 156.12 (C-6); 150.52 (C-4); 139.47 (C-8); 113.10 (C-5); 56.18 (C-2′); 47.58 (C-1′); 23.35 (N–CH); 6.74, 2C (CH_2). UV: 296.10 (12.15), 272.90 (6.79), 255.60 (10.97).

3.2.8. 2-Amino-3-[2-amino-6-(pyrrolidin-1-yl)purin-9-yl]propanoic acid (4h). Crystallization from water afforded white solid (42%), mp 245 °C dec., E_{Up} = 0.33. For $C_{12}H_{17}N_7O_2{\cdot}H_2O$ (309.32) calcd: C, 47.99; H, 6.04; N, 32.65; O, 13.32. Found: C, 47.79; H, 6.05; N, 32.48. FAB MS: 292 (MH^+) (100). ^{1}H NMR (D_2O + NaOD): 7.50 (s, 1H, H-8); 4.22 (dd, 1H, $J(1'a,2')$ = 5.2, $J(gem)$ = 14.2, H-1′a); 4.00 (dd, 1H, $J(1'b,2')$ = 7.8, $J(gem)$ = 14.2, H-1′b); 3.62 (dd, 1H, $J(2',1'a)$ = 5.2, $J(2',1'b)$ = 7.8, H-2′); 3.62 and 3.33 (2 × m, 2 × 2H, N–CH_2); 1.93 and 1.87 (2 × m, 2 × 2H, CH_2). ^{13}C NMR (D_2O + NaOD): 179.29 (C=O); 159.46 (C-2); 152.71 (C-6); 150.94 (C-4); 138.30 (C-8); 113.31 (C-5); 56.10 (C-2′); 48.60 and 47.50 (N–CH_2); 47.55 (C-1′); 25.53 and 23.74 (CH_2). UV: 298.10 (13.02), 274.40 (7.06), 257.40 (10.86).

3.2.9. 2-Amino-3-(guanin-9-yl)propanoic acid (4i). Crystallized from water to give white solid (18%), mp 230 °C dec., E_{Up} = 0.54. For $C_8H_{10}N_6O_3{\cdot}4/3H_2O$ (262.20) calcd: C, 37.50; H, 4.72; N, 32.80; O, 24.98. Found: C, 37.31; H, 4.83; N, 32.64. FAB MS: 239 (MH^+) (54). ^{1}H NMR (D_2O + NaOD): 7.64 (s, 1H, H-8); 4.29 (dd, 1H, $J(1'a, 2')$ = 5.4, $J(gem)$ = 14.4, H-1′a); 4.12 (dd, 1H, $J(1'b,2)$ = 7.4, J (gem) = 14.4, H-1′b); 3.71 (dd, 1H, $J(2',1'a)$ = 5.4, $J(2',1'b)$ = 7.4,H-2′). ^{13}C NMR (D_2O + NaOD): 179.55 (C=O); 168.31 (C-6); 161.17 (C-2); 151.17 (C-4); 138.58 (C-8); 117.51 (C-5); 56.21 (C-2); 47.60 (C-1″). UV: 278.00 (9.80), 253.80 (14.39), 226.40 (3.79), 197.60 (24.24).

3.2.10. 2-Amino-3-(2-amino-6-sulfanylpurin-9-yl)propanoic acid (4j). Crystallized from water with addition of acetic acid (pH 4), pale yellow solid (21%), mp 253–254°C dec., E_{Up} = 0.60. For $C_8H_{10}N_6O_2S{\cdot}H_2O$ (272.28) calcd: C, 35.29; H, 4.44; N, 30.87; O, 17.63; S, 11.77. Found: C, 35.00; H, 4.51; N, 30.64; S, 12.01. FAB MS: 255.1 (MH^+) (13). ^{1}H NMR (D_2O + NaOD): 7.70 (s, 1H, H–8); 4.30 (dd, 1H, $J(1'a,2')$ = 5.4, $J(gem)$ = 14.2, H-1′a); 4.14 (dd, 1H, $J(1'b,2'')$ = 7.5, $J(gem)$ = 14.2, H-1′b); 3.70 (dd, 1H, $J(2',1'a)$ = 5.4, $J(2',1''b)$ = 7.5, H-2′). ^{13}C NMR (D_2O + NaOD): 179.49 (C-6); 177.90 (C=O); 158.80 (C-2); 148.23 (C-4); 140.60 (C-8); 129.41 (C-5); 56.12 (C-2′); 47.60 (C-1′). UV: 342.20 (17.32), 294.60 (0.44), 261.00 (6.10), 241.60 (1.08), 207.80 (18.25).

3.3. Source, isolation, and cultivation of cells

In vitro biological assays were performed using mouse resident peritoneal macrophages. Female mice of the inbred strain C57BL/6, 8–10 weeks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set on from 6 a.m. to 6 p.m., temperature at 22 °C. Animals, killed by cervical dislocation, were ip injected with 8 mL of sterile saline. Pooled peritoneal cells collected from mice (n = 4–8 in individual experiments) were washed, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar, Cambridge, MA) in 100-μL volumes, 2×10^5 cells/well. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells. Cultures were maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator for 24 h. All protocols were approved by the Institutional Ethics Committee.

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

3.4. Nitric oxide (NO) assay

The cells were cultured for 24 h in the presence of test compounds ± murine recombinant interferon-γ (IFN-γ; R&D Systems, Minneapolis, MN). The concentration of nitrites in supernatants of cells was taken as a measure of NO production.[22] It was detected in individual, cell-free samples (50 μl) incubated for 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanylamide/0.1% naphthylenediamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to nitrite concentration.

3.5. Chemokine assays

Macrophages were cultured for 5 h in the absence or presence of test compounds. Concentration of chemokines RANTES (CCL5) and MIP-1α (CCL3) in cell supernatants was determined using enzyme-linked immunoabsorbent assay (ELISA) kits following the manufacturer's instructions (R&D Systems).

3.6. Statistical analysis

Analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA). All data were reported as means ± SEM.

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