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Aminomalononitrile inspired prebiotic chemistry as a novel multicomponent tool for the synthesis of imidazole and purine derivatives with anti-influenza A virus activity†

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Amino imidazole carbonitrile derivatives decorated with α -amino acid side-chains have been synthesized by a multicomponent microwave assisted reaction inspired by the prebiotic chemistry of aminomalononitrile as a tool for generating high chemical diversity. These compounds were used as annulation synthons for the preparation of 8,9-disubstituted-6,9-dihydro-1*H*-purin-6-ones by reaction with formic acid as a simple C-1 donor reagent. The novel heterocycles were characterized by significant activity against influenza A virus, amino imidazole carbonitrile derivatives showing the highest activity. Thus, the chemical complexity generated by prebiotic chemistry furnished a general tool for the identification of novel antiviral agents.

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Introduction

It is well recognized that Multi-Component Chemistry (MCC) played a key role in molecular evolution generating high chemical diversity from a limited number of reagents.¹ The MCC of the Origins of Life generated an incredible variety of organic structures in the pristine Earth, setting the molecular evolution conditions for the emergence of the Last Universal Common Ancestor (LUCA).² The first documented prebiotic MCC encompasses the Strecker reaction for the synthesis of amino acids,³ the Butlerow condensation of formaldehyde to sugars,⁴ and the Orò transformation of cyanuric acid (HCN) to purine nucleobases.⁵ More recently, prebiotic MCC involving formamide (NH₂CHO), a product of hydrolysis of HCN,⁶ yielded a robust chemical framework for the synthesis of sugars, carboxylic acids, amino acids and heterocyclic compounds, including purine and pyrimidine nucleobases and nucleosides.^{7–13} A number of unnatural derivatives were also synthesized by the combination of the initial products, including

peptide nucleic acids¹⁴ and modified acyclic nucleosides,¹⁵ which belong to well-known antiviral drug families.¹⁶ In particular, peptide nucleic acid derivatives are characterized by high antiviral activity against influenza A, HIV and HBV viruses.^{17,18} Today, the modernization of the MCC by innovative techniques (*e.g.* microwave-assisted procedures) furnishes an impressive combination of tools to force and speed-up the production of complex heterocyclic derivatives encompassing the so called “Molecular Evolution-Inspired Synthesis”.¹⁹ In this context, aminomalononitrile (AMN)²⁰ has been identified as a common intermediate in the prebiotic chemistry of HCN and NH₂CHO, besides to diaminomaleonitrile.^{21,22} AMN is involved in the formation of imidazole intermediates, such as amino imidazole carbonitrile and amino imidazole carboxamide, which are precursors of purine and pyrimidine nucleobases and analogues.²³ Imidazoles are important pharmacophores in natural products and drugs, including antiviral agents²⁴ with anti-influenza virus activity.^{25–27} In addition, imidazoles are synthons in the preparation of bioactive purines by annulation with compounds as simple as urea, guanidine, formic acid and NH₂CHO.²⁸ Here we report that AMN inspired multicomponent prebiotic chemistry yields amino imidazole carbonitrile derivatives decorated with different α -amino acid side-chains. The reaction involves the microwave-assisted condensation between aminomalononitrile *para*-toluenesulfonate salt (AMNS), α -amino acids and trimethyl orthoacetate (TOA). The chimera between nucleobases and amino acids has been largely explored in the case of peptide-nucleic acids as a possible alternative to RNA in the origin of life scenarios.²⁹ Imidazole derivatives were successively annulated into the corresponding purines by a solvent free microwave

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† Electronic supplementary information (ESI) available: ¹H-NMR and ¹³C-NMR of 4a–f, 5a–f, 6a–f and 7a–f. See DOI: 10.1039/d1ra05240c



assisted pyrimidine ring-closing procedure involving formic acid. Formic acid is a ubiquitous component in the pristine Earth, often involved in the prebiotic chemistry of AMN.²⁹ The novel derivatives showed appreciable activity against influenza A virus as a consequence of the block of the viral budding from the cell, impairing the viral release to other cells.

Results and discussion

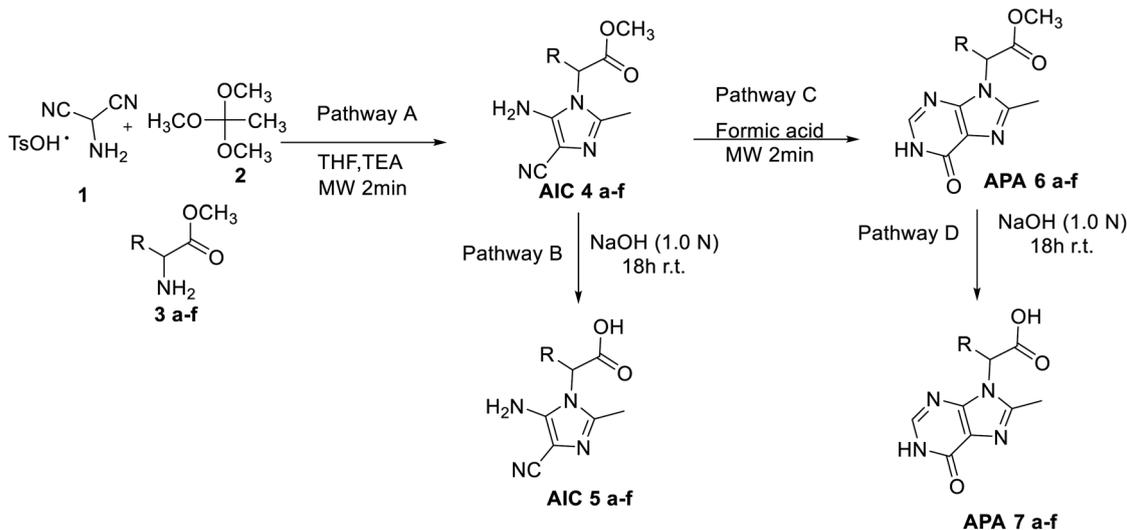
Synthesis of amino imidazole carbonitrile derivatives 4a–f and 5a–f

Amino imidazole carbonitrile derivatives were prepared by a multicomponent procedure involving the microwave assisted condensation of AMNS **1**, TOA **2** and a panel of protected α -amino acids (methyl ester derivatives) **3a–f**, including glycine **3a**, alanine **3b**, valine **3c**, serine **3d**, phenylalanine **3e**, and tyrosine **3f** (Scheme 1). TOA was preferred to trimethyl orthoformate in order to avoid undesired side-processes.³⁰ The reaction was performed by two sequential steps without intermediate workup and purification as follows: (a) AMNS **1** (5.9 mmol) dissolved in THF (30 mL) and triethylamine TEA (7.1 mmol, 1.0 mL) was stirred at room temperature for 30 min, followed by the addition of **2** (8.3 mmol) and microwave irradiation (250 W, 250 psi) for 2.0 min at 200 °C; (b) the solution was cooled down at 25 °C and the crude treated with TEA (7.1 mmol, 1.0 mL) and α -amino acids **3a–f** (7.1 mmol) under previously reported microwave conditions. The reaction proceeded with a quantitative conversion of **1** to afford amino imidazole carbonitrile methyl ester derivatives **4a–f** in a yield ranging from 25% to 60% (Table 1, entries 1–6) depending on the nature of selected α -amino acid (Scheme 1; Pathway A). The closure of the imidazole ring proceeded *via* imino-ether intermediate followed by nucleophilic addition of the amino acid residue.³¹ In this latter case, compounds **4a–f** were selectively decorated by the α -amino acid side-chain at the N-1 position of

the imidazole ring. The possible formation of bis-imidazole derivatives by reaction of compounds **4a–f** with trimethyl orthoformate was not observed under our experimental conditions.²⁸ On the other hand, the low value of the mass balance was probably due to the competitive self-oligomerization of AMN to HCN polymers.³² As a general trend, the yield of the reaction products decreased by increasing the structural complexity of the α -amino acid side-chain (Table 1, entry 1 *vs.* entries 2–3), with the only exception of aromatic derivatives phenyl alanine and tyrosine (Table 1, entries 5–6). The beneficial effect of the aromatic moiety in the annulation of AMN with simple amine has been previously reported.²⁸ Compounds **4a–f** were successively de-protected (NaOH 1 M for 18 h at 25 °C)³³ to afford the corresponding amino imidazole carbonitriles (AIC) **5a–f** (Scheme 1, Pathway B) (Table 1, entries 7–12).

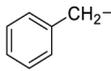
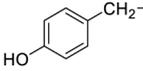
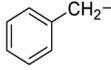
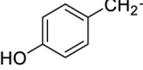
Synthesis of 8,9-disubstituted-6,9-dihydro-1H-purin-6-one derivatives 6a–f and 7a–f

The role of amino imidazole carbonitriles as synthons for the synthesis of a large diversity of heterocyclic derivatives has been reported, and the mechanism of the condensation discussed in detail.^{34–36} For example, C-1 containing reagents as simple as urea, guanidine, formic acid and NH_2CHO , have been applied to yield di-amino purines, hypoxanthine, adenine, 1H-purin-2-(3H)-ones and other analogues, respectively. On the basis of these data, compounds **4a–f** were annulated with formic acid to yield the corresponding 8,9-disubstituted-6,9-dihydro-1H-purin-6-one methyl esters **6a–f**. Briefly, compounds **4a–f** (3.8 mmol) were dissolved in formic acid (3.0 mL) under microwave irradiation (250 W, 250 psi) for 2.0 min at 200 °C (Scheme 1, Pathway C) to afford **6a–f** in quantitative conversion of substrate and yield (Table 2, entries 1–6). In addition, **6a–f** (0.1 mmol) were successively de-protected (NaOH 1 M for 18 h at 25 °C) to corresponding 8,9-disubstituted-6,9-dihydro-1H-purin-6-ones **7a–f** (Scheme 1, Pathway D) (Table 2, entries 7–12).



Scheme 1 Synthesis of amino imidazole carbonitrile derivatives **4a–f** and **5a–f**, and 8,9-disubstituted-6,9-dihydro-1H-purin-6-ones **6a–f** and **7a–f**.

Table 1 Amino imidazole carbonitrile methyl esters **4a–f** and amino imidazole carbonitriles **5a–f** from three component condensation of AMN, trimethyl orthoacetate and α -amino acids

| Entry | α -Amino acid | Condition | Products | R | Yield ^c (%) |
|-------|----------------------|---|-----------|---|------------------------|
| 1 | Glycine | THF, 2.0 min, 250 W, 250 psi at 200 °C ^a | 4a | H | 60 |
| 2 | Alanine | | 4b | CH ₃ | 34 |
| 3 | Valine | | 4c | CH(CH ₃) ₂ | 32 |
| 4 | Serine | | 4d | CH ₂ OH | 25 |
| 5 | Phenylalanine | NaOH, 18 h, 25 °C ^b | 4e |  | 51 |
| 6 | Tyrosine | | 4f |  | 48 |
| 7 | Glycine | | 5a | H | 92 |
| 8 | Alanine | | 5b | CH ₃ | 95 |
| 9 | Valine | | 5c | CH(CH ₃) ₂ | 98 |
| 10 | Serine | | 5d | CH ₂ OH | 93 |
| 11 | Phenylalanine | | 5e |  | 90 |
| 12 | Tyrosine | | 5f |  | 94 |

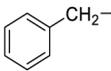
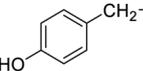
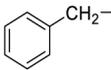
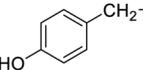
^a Reaction conditions: (a) compound **1** (5.9 mmol) in THF (30 mL) and triethylamine (7.1 mmol) for 30 min at 25 °C; (b) compound **2** (8.3 mmol) and amino-acids **3a–f** (7.1 mmol) under MW irradiation. ^b NaOH (1 N, 1.0 mL) stirring for 18 h at 25 °C. ^c The yield was calculated on the basis of the amount of the recovered product. Reactions were performed in triplicate.

Biological assay

Influenza A virus is one of the most common infectious human respiratory pathogens associated with significant morbidity and

mortality, especially in hospitalized patients. Among the influenza viruses, type A strains are responsible for annual epidemics and pandemic events. They belong to the Orthomyxoviridae family and are characterized by segmented, single-

Table 2 Synthesis of amino purine analogues **6a–f** and **7a–f**

| Entry | α -Amino acid | Condition | Products | R | Yield ^c (%) |
|-------|----------------------|---|-----------|---|------------------------|
| 1 | Glycine | Formic acid, 2.0 min, 250 W, 250 psi at 200 °C ^a | 6a | H | 88 |
| 2 | Alanine | | 6b | CH ₃ | 85 |
| 3 | Valine | | 6c | CH(CH ₃) ₂ | 87 |
| 4 | Serine | | 6d | CH ₂ OH | 92 |
| 5 | Phenylalanine | NaOH, 18 h, 25 °C ^b | 6e |  | 90 |
| 6 | Tyrosine | | 6f |  | 95 |
| 7 | Glycine | | 7a | H | 98 |
| 8 | Alanine | | 7b | CH ₃ | 94 |
| 9 | Valine | | 7c | CH(CH ₃) ₂ | 96 |
| 10 | Serine | | 7d | CH ₂ OH | 92 |
| 11 | Phenylalanine | | 7e |  | 94 |
| 12 | Tyrosine | | 7f |  | 88 |

^a Reaction conditions: compounds **4a–f** (3.8 mmol) were dissolved in formic acid (3.0 mL) under MW irradiation. ^b NaOH (1 N, 1.0 mL) stirring for 18 h at 25 °C. ^c The yield was calculated on the basis of the amount of the recovered product. Reactions were performed in triplicate.



stranded negative RNA genome. RNA segments associate with the nucleoprotein (NP) and the viral RNA-dependent RNA polymerase (RdRp) complex in the replication and transcription cycles.³⁷ Three families of antiviral compounds are active against influenza A virus: (a) the first one blocks the ion-channel activity of the viral matrix (M) 2 protein involved in the virus uncoating; (b) the second family includes inhibitors of the viral neuraminidase (NA), blocking the release of viral particles from infected cells; and (c) inhibitors of the polymerase complex. Among them, baloxavir, marboxil and favirapir are licensed in Japan and United States.³⁸ However, the huge circulation of different influenza viruses and the emergence of drug-resistant strains lead to the search of new antiviral agents. For this reason, the novel imidazole and purine derivatives were tested against influenza A/Puerto Rico/8/34 H1N1 (PR8) virus. In the first set of experiments, A549 cells were infected with 0.001 MOI of PR8 and treated with different concentrations (range 5–80 $\mu\text{g mL}^{-1}$) of compounds **4a–f**, **5a–f**, **6a–f** and **7a–f** (Table 3) for 24 h. Then, infected treated-cells were fixed and stained with antibodies specific for the viral protein hemagglutinin (HA), as described in the experimental part. The cytotoxicity of the compounds was evaluated by standard MTT assay. Table 3 shows the values of IC_{50} , CC_{50} , and relative selective index (SI) obtained on A549 cells. Compounds **4e**, **5a**, **4b**, **4d**, **5e**, **7c**, **6c**, **6f**, and **7b** showed SI values

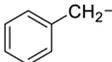
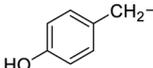
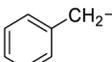
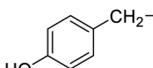
more than 1.5. These compounds were further investigated to evaluate the ability in impairing viral release from infected cells. Briefly, A549 cells were infected with PR8 and treated with concentrations (100, 150 and 200 $\mu\text{g mL}^{-1}$) of selected compounds. After 24 h, infected cell monolayers were directly stained with anti-HA antibodies as described above, while supernatants were recovered and used to newly infect fresh monolayers. We found that within the 9 most active derivatives, compounds **5a** and **7c** decreased the expression of HA on infected monolayers (Fig. 1A) compared to untreated infected cells (Fig. 1; Panel A, part I). The percentage of Relative Fluorescent Units (RFU) of HA was dose-dependently reduced. As a consequence, the supernatants of treated cells contained less viral particles able to infect other monolayers (Fig. 1; Panel B). Overall, the results suggest that both compounds **5a** and **7c** may block specific steps of viral replication, probably involving the viral budding from the cell, thus impairing viral release from infected cells and the spreading of virions to the other ones.

Experimental section

Materials

Solvents and reagents (analytical grade) were purchased from Aldrich Chemical Co. Silica gel 60 F254 plates and silica gel 60

Table 3 Biological activity of compounds **4a–f**, **5a–f**, **6a–f** and **7a–f** against influenza A virus

| Entry | Class | Amino acid | R | Products | IC_{50}^a | CC_{50}^b | SI^c |
|-------|--|--|--|-----------|--------------------|--------------------|---------------|
| 1 | Amino-imidazole-carbonitrile derivatives (AIC) | Glycine | H | 4a | nd | 1.02 | nd |
| 2 | | | | 5a | 0.76 | 2.20 | 2.9 |
| 3 | | Alanine | CH_3 | 4b | 0.76 | 2.75 | 3.6 |
| 4 | | | | 5b | 3.00 | 2.91 | 1 |
| 5 | | Valine | $\text{CH}(\text{CH}_3)_2$ | 4c | 2.41 | 1.13 | 0.5 |
| 6 | | | | 5c | 0.83 | 0.62 | 0.8 |
| 7 | | Serine | CH_2OH | 4d | 0.68 | 1.99 | 2.9 |
| 8 | | | | 5d | nd | nd | nd |
| 9 | | Phenylalanine |  | 4e | 0.39 | 0.84 | 2.1 |
| 10 | | | | 5e | 0.46 | 3.36 | 7.2 |
| 11 | Tyrosine |  | 4f | 3.20 | 1.45 | 0.5 | |
| 12 | | | 5f | 1.99 | 0.90 | 0.45 | |
| 13 | Amino-purine-analogues (APA) | Glycine | H | 6a | 6.96 | 2.11 | 0.3 |
| 14 | | | | 7a | 0.89 | 0.75 | 0.84 |
| 15 | | Alanine | CH_3 | 6b | nd | 0.47 | nd |
| 16 | | | | 7b | 1.85 | 2.86 | 1.5 |
| 17 | | Valine | $\text{CH}(\text{CH}_3)_2$ | 6c | 0.18 | 0.31 | 1.7 |
| 18 | | | | 7c | 0.59 | 1.09 | 1.8 |
| 19 | | Serine | CH_2OH | 6d | nd | 0.93 | nd |
| 20 | | | | 7d | — | 6.11 | — |
| 21 | | Phenylalanine |  | 6e | 1.87 | 0.51 | 0.3 |
| 22 | | | | 7e | nd | 6.35 | nd |
| 23 | | Tyrosine |  | 6f | 0.48 | 0.80 | 1.7 |
| 24 | | | | 7f | nd | nd | nd |

^a IC_{50} is the drug concentration (μmol) causing 50% inhibition of the desired activity. Each experiment was conducted in triplicate. ^b CC_{50} is the drug concentration (μmol) causing 50% of death of viable cell. ^c SI is the selectivity index defined as the ratio of the CC_{50} to the IC_{50} .



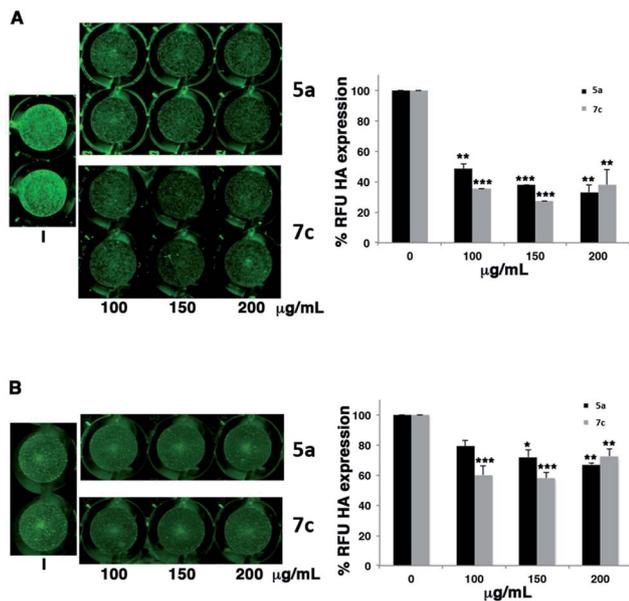


Fig. 1 The expression of hemagglutinin (HA) by means of In Cell Western (ICW) assay in the presence of most active compounds **5a** and **7c**. A549 cells were infected with PR8 (0.001 MOI) and treated or not (I) with different concentrations (100, 150 and 200 $\mu\text{g mL}^{-1}$) of compounds. After 24 h treatment, cells were fixed and stained for HA protein, as described in the Materials and methods section (Panel A); supernatants were recovered and used to infect a fresh monolayer of A549 cells (Panel B). The expression of viral HA was analyzed by ICW assay, using LI-COR Image Studio Software. The percentage of relative fluorescence units (RFU) was calculated in comparison to untreated infected cells (considered 100%). Values are the mean \pm S.D. of two replicates of one experiment of two performed ($n = 2$). Statistical significance of the data vs. untreated infected cells was defined as * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$ (unpaired t-test).

were furnished from Merck. Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates using a UV lamp for visualization. Merck silica gel 60 (230–400 mesh) was used for chromatography. Microwave reactions were performed by a microwave synthesizer CEM Discover (CEM Corporation Italy). NMR spectra were recorded on a Bruker Advance DRX400 (400 MHz/100 MHz) spectrometer. Chemical shifts are reported in parts per million (ppb) using deuterated solvents DMSO- d_6 , CD₃OD, D₂O, CDCl₃. Coupling constants (J) are reported in Hz. Multiplicities are reported in the conventional form: s = singlet, d = doublet, t = triplet, dd = doublets of doublets, m = multiplet. A 549 (human lung epithelial carcinoma) cell lines (ATCC code no. CCL-185) were purchased from LGC Standards Srl, Sesto San Giovanni, Milan Italy. Influenza A/PR/8/34 (H1N1) virus (ATCC code no. VR-95) purchased from LGC Standards Srl, Sesto San Giovanni, Milan Italy.

General procedure for the synthesis of 5-amino-1,2-disubstituted-1H-imidazole-4-carbonitriles (**4a–f**)

To a solution of aminomalononitrile *p*-toluenesulfonate **1** (5.9 mmol) in THF (30 mL) was added triethylamine (7.1 mmol). The mixture was stirred at room temperature for 30 min. To this

solution was added trimethyl orthoacetate **2** (8.3 mmol), and the solution was stirred under microwave condition using the following program:

| No. of cycles | Temperature | Ramp time | Hold time | Pressure (psi) | Power (W) |
|---------------|-------------|-----------|-----------|----------------|-----------|
| 1 | 200 °C | 1 min | 2 min | 250 | 250 |

Thereafter, the solution was cooled at room temperature followed by the addition of triethylamine (7.1 mmol) and amino-acids (methyl ester derivatives) **3a–f** (7.1 mmol). The solution was stirred under previously reported microwave conditions. The solvent was removed under reduced pressure and the residue dissolved in dichloromethane (30 mL) was washed with Na₂CO₃ (3 \times 20 mL) and NaCl (1 \times 20 mL) saturated solutions. The organic layer was dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The crude was purified by flash-chromatography eluting with ethyl acetate : *n*-hexane (2 : 1) to afford **4a–f** from 15 to 60% yield.

General procedure for the synthesis of amino-purine-analogues (**6a–f**)

Compound **4a–f** (3.8 mmol) was dissolved in formic acid (3.0 mL) and stirred under microwave condition described for the synthesis of **4a–f**. After returning to room temperature, the mixture was poured into water (30 mL). The resulting precipitate was filtered and washed with water. The crude was purified by flash-chromatography eluting with dichloromethane (CH₂-Cl₂) : methanol (CH₃OH) (5 : 1) to afford **6a–f** in quantitative yield.

General procedure for the synthesis of amino-purine-analogues (**5a–f** and **7a–f**)

The appropriate compound (0.1 mmol) was hydrolyzed in NaOH solution (1 N, 1.0 mL) stirring for 18 h at room temperature. Thereafter, the solution was neutralized with HCl 1 N, freeze-dried and the residue washed with CH₃OH. The organic layer was evaporated under reduced pressure to afford **5a–f** and **7a–f** in quantitative yield. ¹H-NMR and ¹³C-NMR of **5a–f** and **7a–f** are in ESI#1.†

Spectroscopic data

Compound 4a. Methyl 2-(5-amino-4-cyano-2-methyl-1H-imidazol-1-yl)acetate. ¹H-NMR (400 MHz, DMSO- d_6 , ppm): δ 6.135 (s, 2H, NH₂), 4.733 (s, 2H, CH₂), 3.706 (s, 3H, O-CH₃), 2.078 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO- d_6 , ppm): δ 168.515 (C=O), 148.883 (C), 140.104 (C), 118.080 (C), 88.533 (C), 52.912 (O-CH₃), 43.907 (CH₂), 13.297 (CH₃). MS (ESI): m/z (M + H) +195.19. Elemental analysis for C₈H₁₀N₄O₂ calculated: C, 49.48; H, 5.19; N, 28.85; O, 16.48. Found: C, 49.45; H, 5.16; N, 28.81; O, 16.43.

Compound 4b. Methyl 2-(5-amino-4-cyano-2-methyl-1H-imidazol-1-yl)propanoate. The crude residue was purified by flash-chromatography eluting with ethyl acetate : petroleum ether (2 : 1). ¹H-NMR (400 MHz, CDCl₃, ppm): δ 4.897–4.878 (m,



1H, CH), 4.150 (s, 2H, NH₂), 3.847 (s, 3H, O-CH₃), 2.336 (s, 3H, CH₃), 1.761 (d, *J* = 7.6 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, CDCl₃, ppm): δ 170.573 (C=O), 145.708 (C), 140.664 (C), 115.587 (C), 96.244 (C), 53.373 (CH), 53.297 (O-CH₃), 15.965 (CH₃), 14.096 (CH₃). MS (ESI): *m/z* (M + H) +209.22. Elemental analysis for C₉H₁₂N₄O₂ calculated: C, 51.92; H, 5.81; N, 26.91; O, 15.37. Found: C, 51.89; H, 5.77; N, 26.87; O, 15.32.

Compound 4c. Methyl 2-(5-amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-methylbutanoate. The crude residue was purified by flash-chromatography eluting with ethyl acetate : *n*-hexane (2 : 1). ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 6.042 (s, 2H, NH₂), 4.577 (d, *J* = 10.8 Hz, 1H, CH), 3.705 (s, 3H, O-CH₃), 2.597–2.553 (m, 1H, CH), 2.123 (s, 3H, CH₃), 1.113 (d, *J* = 6.4 Hz, 3H, CH₃), 0.639 (d, *J* = 6.4 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 169.805 (C=O), 148.935 (C), 139.722 (C), 117.724 (C), 89.925 (C), 61.883 (CH), 53.198 (O-CH₃), 28.372 (CH), 20.673 (CH₃), 18.860 (CH₃), 14.716 (CH₃). MS (ESI): *m/z* (M + H) +238.28. Elemental analysis for C₁₁H₁₆N₄O₂ calculated: C, 55.92; H, 6.83; N, 23.71; O, 13.54. Found: C, 55.86; H, 6.78; N, 23.65; O, 13.49.

Compound 4d. Methyl 2-(5-amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-hydroxypropanoate. The crude residue was purified by flash-chromatography eluting with ethyl acetate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 6.890 (s, 1H, OH), 5.872 (s, 2H, NH₂), 5.473–5.092 (m, 2H, CH₂), 3.689 (s, 3H, O-CH₃), 3.666–3.615 (m, 1H, CH), 2.114 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 168.27 (C=O), 148.98 (C), 140.56 (C), 117.99 (C), 90.09 (C), 62.85 (CH₂), 59.86 (CH), 53.09 (O-CH₃), 14.55 (CH₃). MS (ESI): *m/z* (M + H) +225.22. Elemental analysis for C₉H₁₂N₄O₃ calculated: C, 48.21; H, 5.39; N, 24.99; O, 21.41. Found: C, 48.17; H, 5.35; N, 24.94; O, 21.38.

Compound 4e. Methyl 2-(5-amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-phenylpropanoate. The crude residue was purified by flash-chromatography eluting with ethyl acetate : *n*-hexane (3 : 1). ¹H-NMR (400 MHz, CDCl₃, ppm): δ 7.283–7.254 (m, 3H, CH-Ar), 6.995–6.973 (m, 2H, CH-Ar), 4.837–4.799 (dd, *J* = 4.0, 11.2 Hz, 1H, CH₂), 4.323 (s, 2H, NH₂), 3.877 (s, 3H, O-CH₃), 3.569 (t, *J* = 12.8 Hz, 1H, CH), 3.385–3.340 (dd, *J* = 4.4, 13.6 Hz, 1H, CH₂), 1.809 (s, 3H, CH₃). ¹³C-NMR (100 MHz, CDCl₃, ppm): δ 169.934 (C=O), 146.094 (C), 141.435 (C), 135.347 (C), 129.080 (C-Ar × 2), 128.757 (C-Ar × 2), 127.768 (C-Ar), 115.833 (C), 95.757 (C), 60.286 (CH), 53.498 (O-CH₃), 35.296 (CH₂), 13.456 (CH₃). MS (ESI): *m/z* (M + H) +285.32. Elemental analysis for C₁₅H₁₆N₄O₂ calculated: C, 63.37; H, 5.67; N, 19.71; O, 11.25. Found: C, 63.31; H, 5.62; N, 19.68; O, 11.20.

Compound 4f. Methyl 2-(5-amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-(4-hydroxyphenyl)propanoate. The crude residue was purified by flash-chromatography eluting with ethyl acetate : petroleum ether (4 : 1). ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 9.249 (s, 1H, OH), 6.846 (d, *J* = 8.4 Hz, 2H, CH-Ar), 6.593 (d, *J* = 8.4 Hz, 2H, CH-Ar), 5.700 (s, 2H, NH₂), 5.199–5.160 (m, 1H, CH), 3.724 (s, 3H, O-CH₃), 3.266–3.219 (m, 2H, CH₂), 1.794 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 169.44 (C=O), 156.59 (C-OH), 148.22 (C), 140.24 (C), 130.41 (C-Ar × 2), 126.71 (C-Ar), 117.83 (C), 115.60 (C-Ar × 2), 90.92 (C), 60.22 (CH), 53.26 (O-CH₃), 34.19 (CH₂), 14.56 (CH₃). MS (ESI): *m/z* (M + H) +301.32. Elemental analysis for C₁₅H₁₆N₄O₃ calculated: C,

59.99; H, 5.37; N, 18.66; O, 15.98. Found: C, 59.95; H, 5.29; N, 18.61; O, 15.94.

Compound 5a. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)acetic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 4.285 (d, *J* = 8.8 Hz, 2H, CH₂), 2.112 (d, *J* = 11.2 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 174.50 (C=O), 144.50 (C), 141.60 (C), 109.61 (C), 95.61 (C), 45.88 (CH₂), 12.00 (CH₃). MS (ESI): *m/z* (M + H) +181.17. Elemental analysis for C₇H₈N₄O₂ calculated: C, 46.67; H, 4.48; N, 31.10; O, 17.76. Found: C, 46.62; H, 4.43; N, 31.05; O, 17.71.

Compound 5b. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)propanoic acid. ¹H-NMR (400 MHz, MeOD, ppm): δ 4.260 (d, *J* = 7.6 Hz, 1H, CH), 1.747 (s, 3H, CH₃), 1434 (s, 3H, CH₃). ¹³C-NMR (100 MHz, MeOD, ppm): δ 167.11 (C=O), 142.78 (C), 139.26 (C), 117.28 (C), 93.45 (C), 58.05 (CH), 22.48 (CH₃), 14.60 (CH₃). MS (ESI): *m/z* (M + H) +195.19. Elemental analysis for C₈H₁₀N₄O₂ calculated: C, 49.48; H, 5.19; N, 28.85; O, 16.48. Found: C, 49.43; H, 5.15; N, 28.79; O, 16.41.

Compound 5c. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-methylbutanoic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 4.384 (d, *J* = 2.8 Hz, 1H, CH), 2.409–2.340 (m, 1H, CH), 2.229 (s, 3H, CH₃), 0.937 (d, *J* = 6.8 Hz, 3H, CH₃), 0.756 (d, *J* = 6.8 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 181.50 (C=O), 148.33 (C), 141.22 (C), 117.46 (C), 92.58 (C), 67.34 (CH), 29.76 (CH), 16.76 (CH₃), 16.17 (CH₃), 13.25 (CH₃). MS (ESI): *m/z* (M + H) +223.25. Elemental analysis for C₁₀H₁₄N₄O₂ calculated: C, 54.04; H, 6.35; N, 25.21; O, 14.40. Found: C, 53.97; H, 6.31; N, 25.17; O, 14.35.

Compound 5d. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-hydroxypropanoic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 4.489–4.415 (m, 1H, CH), 3.119 (s, 2H, CH₂), 2.625 (s, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 168.62 (C=O), 145.80 (C), 140.76 (C), 116.44 (C), 94.79 (C), 64.43 (CH₂), 61.41 (CH), 12.92 (CH₃). MS (ESI): *m/z* (M + H) +211.19. Elemental analysis for C₈H₁₀N₄O₃ calculated: C, 45.71; H, 4.80; N, 26.66; O, 22.83. Found: C, 45.66; H, 4.75; N, 26.61; O, 22.78.

Compound 5e. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-phenylpropanoic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 7.160–7.129 (m, 3H, CH-Ar), 6.930–6.925 (m, 2H, CH-Ar), 3.405–3.358 (dd, *J* = 4.4, 14.4 Hz, 2H, CH₂), 3.204 (d, *J* = 14.4 Hz, 1H, CH), 2.275 (s, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 166.90 (C=O), 154.94 (C), 139.62 (C), 134.76 (C), 129.24 (C-Ar × 2), 128.38 (C-Ar × 2), 127.08 (C-Ar), 108.66 (C), 95.90 (C), 60.23 (CH), 34.70 (CH₂), 12.80 (CH₃). MS (ESI): *m/z* (M + H) +271.29. Elemental analysis for C₁₄H₁₄N₄O₂ calculated: C, 62.21; H, 5.22; N, 20.73; O, 11.84. Found: C, 62.16; H, 5.28; N, 20.69; O, 11.79.

Compound 5f. 2-(5-Amino-4-cyano-2-methyl-1*H*-imidazol-1-yl)-3-(4-hydroxyphenyl)propanoic acid. ¹H-NMR (400 MHz, MeOD, ppm): δ 6.797 (d, *J* = 2.0 Hz, 2H, CH-Ar), 6.494 (d, *J* = 6.4 Hz, 2H, CH-Ar), 4.004–3.961 (m, 1H, CH), 2.885–2.848 (m, 2H, CH₂), 1.9876 (s, 3H, CH₃). ¹³C-NMR (100 MHz, MeOD, ppm): δ 167.02 (C=O), 156.01 (C-OH), 147.00 (C), 137.88 (C), 129.61 (C-Ar × 2), 120.47 (C-Ar), 118.54 (C-Ar × 2), 108.95 (C), 95.90 (C), 59.81 (CH), 35.99 (CH₂), 12.47 (CH₃). MS (ESI): *m/z* (M + H) +287.29. Elemental analysis for C₁₄H₁₄N₄O₃ calculated: C,



58.74; H, 4.93; N, 19.57; O, 16.77. Found: C, 58.69; H, 4.88; N, 19.52; O, 16.71.

Compound 6a. Methyl 2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)acetate. ¹H-NMR (400 MHz, D₂O, ppm): δ 8.067 (s, 1H, CH-Ar), 5.051 (s, 2H, CH₂), 3.744 (s, 3H, O-CH₃), 2.438 (s, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 169.76 (C=O), 155.96 (C), 151.77 (C), 149.80 (C), 145.45 (C), 122.02 (C), 53.42 (O-CH₃), 43.91 (CH₂), 12.49 (CH₃). MS (ESI): *m/z* (M + H) +221.20. Elemental analysis for C₉H₁₀N₄O₃ calculated: C, 48.65; H, 4.54; N, 25.21; O, 21.60. Found: C, 48.61; H, 4.48; N, 25.16; O, 21.55.

Compound 6b. Methyl 2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 8.159 (s, 1H, CH-Ar), 4.714–4.697 (m, 1H, CH), 3.652 (s, 3H, O-CH₃), 2.265 (s, 3H, CH₃), 1.506 (d, *J* = 7.2 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 169.21 (C=O), 163.66 (C), 156.83 (C), 145.26 (C), 136.91 (C), 111.34 (C), 54.79 (O-CH₃), 50.28 (CH), 15.96 (CH₃), 13.40 (CH₃). MS (ESI): *m/z* (M + H) +237.23. Elemental analysis for C₁₀H₁₂N₄O₃ calculated: C, 50.84; H, 5.12; N, 23.72; O, 20.32. Found: C, 50.79; H, 5.06; N, 23.67; O, 20.27.

Compound 6c. Methyl 3-methyl-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)butanoate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 7.970 (s, 1H, CH-Ar), 4.970 (d, *J* = 10.0 Hz, 1H, CH), 3.629 (s, 3H, O-CH₃), 2.851–2.793 (m, 1H, CH), 2.268 (s, 3H, CH₃), 1.120 (d, *J* = 6.8 Hz, 3H, CH₃), 0.744 (d, *J* = 6.8 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 175.20 (C=O), 163.69 (C), 145.67 (C), 138.57 (C), 136.98 (C), 111.13 (C), 63.61 (CH), 52.92 (O-CH₃), 29.96 (CH), 17.52 (CH₃), 16.57 (CH₃), 13.88 (CH₃). MS (ESI): *m/z* (M + H) +265.29. Elemental analysis for C₁₂H₁₆N₄O₃ calculated: C, 54.54; H, 6.10; N, 21.20; O, 18.16. Found: C, 54.49; H, 6.05; N, 21.16; O, 18.11.

Compound 6d. Methyl 3-hydroxy-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 8.090 (s, 1H, CH-Ar), 5.473–5.092 (m, 2H, CH₂), 3.689 (s, 3H, O-CH₃), 3.666–3.615 (m, 1H, CH), 2.141 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 168.07 (C=O), 162.93 (C), 150.65 (C), 146.34 (C), 143.51 (C), 115.97 (C), 63.38 (CH), 61.55 (CH₂), 52.59 (O-CH₃), 13.11 (CH₃). MS (ESI): *m/z* (M + H) +253.23. Elemental analysis for C₁₀H₁₂N₄O₄ calculated: C, 47.62; H, 4.80; N, 22.21; O, 25.37. Found: C, 47.57; H, 4.76; N, 22.18; O, 25.32.

Compound 6e. Methyl 2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)-3-phenylpropanoate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 8.147 (s, 1H, CH-Ar), 7.223–7.197 (m, 3H, CH-Ar), 7.030–7.006 (m, 2H, CH-Ar), 5.068 (t, *J* = 4.8 Hz, 1H, CH), 3.689 (s, 3H, O-CH₃), 3.238 (dd, *J* = 6.2, 13.6 Hz, 2H, CH₂), 2.239 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 175.70 (C=O), 164.18 (C), 147.50 (C), 143.35 (C), 137.09 (C), 135.10 (C), 129.86 (C-Ar × 2), 128.82 (C-Ar × 2), 127.58 (C-Ar), 111.35 (C), 59.98 (CH), 53.16 (O-CH₃), 35.30 (CH₂), 13.72 (CH₃). MS (ESI): *m/z* (M + H) +314.33. Elemental analysis for C₁₆H₁₆N₄O₃ calculated: C, 61.53; H, 5.16; N, 17.94; O, 15.37. Found: C, 61.51; H, 5.12; N, 17.89; O, 15.34.

Compound 6f. Methyl 3-(4-hydroxyphenyl)-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoate. ¹H-NMR (400 MHz, DMSO-*d*₆, ppm): δ 8.142 (s, 1H, CH-Ar), 6.789 (d, *J* = 8.4 Hz, 2H,

CH-Ar), 6.576 (d, *J* = 8.4 Hz, 2H, CH-Ar), 4.962 (t, *J* = 4.8 Hz, 1H, CH), 3.724 (s, 3H, O-CH₃), 3.094 (dd, *J* = 4.4, 10.4 Hz, 2H, CH₂), 2.211 (s, 3H, CH₃). ¹³C-NMR (100 MHz, DMSO-*d*₆, ppm): δ 175.71 (C=O), 163.64 (C), 156.81 (C), 149.99 (C), 144.34 (C), 138.70 (C), 137.05 (C-Ar), 130.80 (C-Ar × 2), 124.93 (C-Ar × 2), 115.62 (C), 60.25 (CH), 54.42 (O-CH₃), 34.56 (CH₂), 13.72 (CH₃). MS (ESI): *m/z* (M + H) +329.33. Elemental analysis for C₁₆H₁₆N₄O₃ calculated: C, 61.53; H, 5.16; N, 17.94; O, 15.37. Found: C, 61.48; H, 5.11; N, 17.89; O, 15.32.

Compound 7a. 2-(8-Methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)acetic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 7.944 (s, 1H, CH-Ar), 4.556 (s, 2H, CH₂), 2.325 (s, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 174.45 (C=O), 166.79 (C), 152.78 (C), 150.80 (C), 149.80 (C), 121.56 (C), 45.46 (CH₂), 12.61 (CH₃). MS (ESI): *m/z* (M + H) +209.18. Elemental analysis for C₈H₈N₄O₃ calculated: C, 46.16; H, 3.87; N, 26.91; O, 23.06. Found: C, 46.11; H, 3.82; N, 26.86; O, 23.00.

Compound 7b. 2-(8-Methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 8.326 (s, 1H, CH-Ar), 5.034–4.978 (m, 1H, CH), 2.180 (s, 3H, CH₃), 1.386 (s, 3H, CH₃). ¹³C-NMR (100 MHz, MeOD, ppm): δ 169.10 (C=O), 160.03 (C), 155.38 (C), 151.92 (C), 137.16 (C), 109.13 (C), 51.58 (CH), 15.11 (CH₃), 11.94 (CH₃). MS (ESI): *m/z* (M + H) +223.20. Elemental analysis for C₉H₁₀N₄O₃ calculated: C, 48.65; H, 4.54; N, 25.21; O, 21.60. Found: C, 48.61; H, 4.49; N, 25.17; O, 21.54.

Compound 7c. 3-Methyl-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)butanoic acid. ¹H-NMR (400 MHz, D₂O, ppm): δ 7.857 (s, 1H, CH-Ar), 4.401 (d, *J* = 10.8 Hz, 1H, CH), 2.236–2.202 (m, 1H, CH), 2.087 (s, 3H, CH₃), 0.786 (d, *J* = 6.8 Hz, 3H, CH₃), 0.614 (d, *J* = 6.8 Hz, 3H, CH₃). ¹³C-NMR (100 MHz, D₂O, ppm): δ 175.56 (C=O), 167.15 (C=O), 152.73 (C), 149.48 (C), 139.90 (C), 108.48 (C), 65.44 (CH), 29.60 (CH), 16.99 (CH₃), 16.20 (CH₃), 13.29 (CH₃). MS (ESI): *m/z* (M + H) +251.26. Elemental analysis for C₁₁H₁₄N₄O₃ calculated: C, 52.79; H, 5.64; N, 22.39; O, 19.18. Found: C, 52.73; H, 5.58; N, 22.33; O, 19.13.

Compound 7d. 3-Hydroxy-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoic acid. ¹H-NMR (400 MHz, CD₃OD, ppm): δ 8.563 (s, 1H, CH-Ar), 5.422–5.404 (m, 2H, CH₂), 3.625–3.500 (m, 1H, CH), 1.190 (s, 3H, CH₃). ¹³C-NMR (100 MHz, CD₃OD, ppm): δ 165.54 (C=O), 155.45 (C), 149.92 (C), 145.68 (C), 142.10 (C), 116.06 (C), 65.92 (CH), 62.67 (CH₂), 14.65 (CH₃). MS (ESI): *m/z* (M + H) +239.07. Elemental analysis for C₉H₁₀N₄O₄ calculated: C, 45.38; H, 4.23; N, 23.52; O, 26.87. Found: C, 45.32; H, 4.19; N, 23.47; O, 26.82.

Compound 7e. 2-(8-Methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)-3-phenylpropanoic acid. ¹H-NMR (400 MHz, CD₃OD, ppm): δ 8.557 (s, 1H, CH-Ar), 7.183–7.120 (m, 5H, CH-Ar), 4.620 (t, *J* = 4.8 Hz, 1H, CH), 3.489 (dd, *J* = 14.4, 13.6 Hz, H, CH₂), 2.146 (s, 3H, CH₃). ¹³C-NMR (100 MHz, CD₃OD, ppm): δ 174.11 (C=O), 166.77 (C), 155.80 (C), 147.79 (C), 137.50 (C), 135.78 (C), 129.15 (C-Ar × 2), 127.98 (C-Ar × 2), 126.54 (C-Ar), 109.04 (C), 66.73 (CH), 35.65 (CH₂), 12.28 (CH₃). MS (ESI): *m/z* (M + H) +299.30. Elemental analysis for C₁₅H₁₄N₄O₃ calculated: C, 60.40; H, 4.73; N, 18.78; O, 16.09. Found: C, 60.35; H, 4.68; N, 18.71; O, 16.01.

Compound 7f. 3-(4-Hydroxyphenyl)-2-(8-methyl-6-oxo-1,6-dihydro-9H-purin-9-yl)propanoic acid. ¹H-NMR (400 MHz, D₂O,



ppm): δ 8.349 (s, 1H, CH-Ar), 6.640 (d, $J = 8.0$ Hz, 2H, CH-Ar), 6.325 (d, $J = 8.0$ Hz, 2H, CH-Ar), 5.150–5.145 (m, 1H, CH), 3.225 (dd, $J = 4.4, 14.8$ Hz, 2H, CH₂), 2.014 (s, 3H, CH₃). ¹³C-NMR (100 MHz, CD₃OD, ppm): δ 174.77 (C=O), 169.08 (C), 151.65 (C), 148.17 (C), 137.93 (C), 129.59 (C-Ar $\times 2$), 128.89 (C-Ar), 120.59 (C), 118.55 (C-Ar $\times 2$), 108.94 (C), 63.40 (CH), 36.14 (CH₂), 12.38 (CH₃). MS (ESI): m/z (M + H) +315.30. Elemental analysis for C₁₅H₁₄N₄O₄ calculated: C, 57.32; H, 4.49; N, 17.83; O, 20.36. Found: C, 57.27; H, 4.43; N, 17.77; O, 20.31.

Cell cultures

A 549 (human lung epithelial carcinoma) cell lines were grown in DMEM 1640 medium supplemented with 10% fetal calf serum (FCS); glutamine 0.3 mg mL⁻¹; penicillin 100 U mL⁻¹ and streptomycin 100 mg mL⁻¹. Cell viability was estimated by trypan blue (0.02%) exclusion. All reagents were purchased from Invitrogen (Milan, Italy).

Cell toxicity assays

The cytotoxicity of the compounds was evaluated by the inhibition of MTT reduction into formazan and by the trypan blue staining assay.³⁹ Briefly, in the MTT assay, A549 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in 100 μ L of complete DMEM without phenol red for 24 h at 37 °C. Subsequently, cell monolayers were treated or not with increasing concentrations (range 5–80 μ g mL⁻¹) of compounds for 24 h at 37 °C. After 24 h, 10 μ L of MTT solution (5 mg mL⁻¹) were added to each well for 3–4 h at 37 °C. Each sample was then acidified by adding 0.1 N HCl in isopropanol (100 μ L per well) for 30 min under mild agitation to ensure dissolution of all formazan crystals. Absorbance of samples was read at 570 nm, using an automatic plate reader (Multiskan EX, Ascent Software, Thermo Fisher Scientific). Untreated cells were used as control. The 50% cytotoxic concentration (CC₅₀) was defined as the compound concentration required to reduce cell viability by 50% and calculated by regression analysis considering untreated cells as control (100%).

Virus production and infection

Influenza A/Puerto Rico/8/34 H1N1 (PR8) virus was grown in the allantoic cavities of 10 day-old embryonated chicken eggs. After 48 h at 37 °C, the allantoic fluid was harvested, centrifuged at 5000 rpm for 30 min to remove cellular debris, and stored at –80 °C. Virus titration was performed by Tissue Culture Infectious Dose 50% (TCID₅₀). Confluent monolayers of A549 epithelial cells were challenged for 1 h at 37 °C with PR8 at a multiplicity of infection (MOI) of 0.001 (TCID₅₀/cell) incubated for 1 h at 37 °C, washed with PBS, and then incubated with medium supplemented with 2% FCS. Mock infection was performed with the same dilution of allantoic fluid from uninfected eggs.⁴⁰

In cell western (ICW) assay

The ICW assay was performed using the Odyssey Imaging System (LI-COR, Lincoln, NE, USA) as previously described.^{41,42}

Briefly, A549 cells grown in 96-well plates (2×10^4 cells per well) infected with PR8, and treated or not with high concentrations (100, 150 and 200 μ g mL⁻¹) of selected compounds were fixed with 4% formaldehyde, washed, permeabilized with 0.1% Triton X-100 and incubated with Odyssey Blocking Buffer for 1 h (LI-COR Biosciences, Lincoln, NE, USA). The cells were then stained at 4 °C overnight with mouse anti HA (Santa Cruz Biotechnology, Germany) diluted in Odyssey Blocking Buffer. Cells were then washed and stained with a fluorochrome-conjugated secondary antibody (fluorescence emission at 800 nm) (LI-COR Biosciences, Lincoln, NE, USA) properly diluted in Odyssey Blocking Buffer. Subsequently, three washes with PBS plus 0.1% Tween 20 were performed and plates were analyzed by the Odyssey infrared imaging system (LI-COR). The relative fluorescence unit (RFU) was expressed as a percentage compared to untreated infected cells (100%). The 50% infectious dose (IC₅₀) was defined as the dose of compound required to reduce viral replication by 50%. The Selectivity Index (SI) of each compound was calculated as the ratio between CC₅₀ and IC₅₀ (SI = CC₅₀/IC₅₀).

Conclusions

Aminomalononitrile inspired prebiotic chemistry furnished an efficient synthetic tool for the synthesis of a large panel of imidazole and purine derivatives decorated with a natural amino acid side-chain motif. These products resemble the first heterocycle derivatives possibly produced by an original multi-component reaction involving well-recognized prebiotic precursors, such as HCN oligomers and amino acids. The annulation of amino-imidazole-carbonitrile derivatives into the corresponding purine analogues generally reduced the activity against influenza A/Puerto Rico/8/34 H1N1 (PR8) virus, suggesting the key role of the imidazole scaffold. The derivatives bearing a free carboxylic moiety in the amino acid residue (compounds **5** and **7**) showed an IC₅₀ value of the same order of magnitude than the corresponding esters (compounds **4** and **6**). The comparable antiviral activity between imidazole derivatives bearing a free or a protected carboxyl moieties has been previously reported in the case of HSV.⁴³ Derivatives decorated with the valine and phenylalanine structural motif showed the higher activity in both AIC and APA series, compounds **4b**, **4e**, **5a**, **5e**, **6c**, **6f** and **7a** being characterized by the highest IC₅₀ value (Table 3, entries 2, 3, 9, 10, 14, 17, 23). Among the AIC family, the presence of the phenylalanine residue produced the most active derivatives, namely compounds **4e** and **5e** (IC₅₀ values of 0.39 μ M and 0.46 μ M), respectively. They also showed the highest selectivity index (2.1 and 7.2, respectively). The valine derivatives **6c** and **7c** were the most active compounds in the APA series, showing IC₅₀ value of 0.18 μ M and 0.59 μ M, respectively, and SI value of 1.7 and 1.8, respectively. Compounds **4b**, **4e**, **5a**, **5e**, **6c**, **6f** and **7a** showed an antiviral activity against influenza A/Puerto Rico/8/34 H1N1 (PR8) virus higher than standard antiviral agents (oseltamivir phosphate, ribavirin and amantadine) and a selectivity index comparable to ribavirin (5.84) and higher than amantadine.⁴⁴ In addition, compounds **5a** and **7c** decreased the expression of viral HA on



infected monolayers, suggesting the occurrence of inhibition of the viral budding from the cell, thus preventing the viral release from infected cells and the spreading to other cells. This hypothesis is in accordance with previous data on the capacity of imidazole derivatives to block the release of virions from infected cells.⁴⁵

The peptide nucleic acid building blocks we synthesized are also relevant to prebiotic research, suggesting novel structural motifs to be considered in pristine molecular evolution processes.

Conflicts of interest

There are no conflicts to declare.

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