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9-Benzoyl 9-deazaguanines as potent xanthine oxidase inhibitors

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ABSTRACT

A novel potent xanthine oxidase inhibitor, 3-nitrobenzoyl 9-deazaguanine (**LSPN451**), was selected from a series of 10 synthetic derivatives. The enzymatic assays were carried out using an on-flow bidimensional liquid chromatography (2D LC) system, which allowed the screening, the measurement of the kinetic inhibition constant and the characterization of the inhibition mode. This compound showed a non-competitive inhibition mechanism with more affinity for the enzyme–substrate complex than for the free enzyme, and inhibition constant of 55.1 ± 9.80 nM, about thirty times more potent than allopurinol. Further details of synthesis and enzymatic studies are presented herein.

1. Introduction

The screening of potential enzymatic inhibitors in synthetic or natural libraries is an important step in drug discovery and development process. Besides its inhibitory potency, to require lower doses to achieve the desired effect, these compounds should exhibit a high degree of selectivity toward the target enzyme.

One of the target enzymes of considerable interest in human therapy is xanthine oxidase (XO). This enzyme participates in the purines metabolism and catalyzes the uric acid production using xanthine and hypoxanthine as substrates. In parallel, the reactive species of oxygen (ROS) are produced, which has been associated with some pathological conditions such as post-ischemic reperfusion injury, diabetes and chronic heart failure.[1] Therefore, the selective inhibition of XO may result in broad-spectrum chemotherapeutic for gout, cancer, inflammation and oxidative damage. Allopurinol is the first clinically used XO inhibitor although causes many side effects such as renal toxicity due to impairment of pyrimidine metabolism.[2] Thus there is a great interest for the development of novel XO inhibitors.[3]

Additionally, XO is frequently used in coupled enzymatic assay to screen purine nucleoside phosphorylase (PNP) inhibitors, as described elsewhere by Kalckar.[4] PNP enzyme is also associated with purine metabolism and catalyzes the cleavage of (deoxy)ribonucleosides, like inosine, in the presence of inorganic phosphate (Pi) to the corresponding purine bases and ribose(deoxyribose)-1-phosphate. Human PNP has been considered an important target to the development of new drugs to treat severe T-cell mediated disorders. In this assay, XO is used to oxidize the product of PNP catalysis (hypoxanthine) producing uric acid that is spectrophotometrically monitored at 293 nm. For PNP, false positive results may be obtained by this approach, thus the recognition of which enzyme is inhibited is high-priority.[5]

To overcome this problem, new models of inhibitors screening assays are necessary and, we have been working on this subject.[6–8] The use of enzymatic bioreactors on a 2D LC system furnishes advantage over other approaches as it allows the screening and characterization of ligands in automatized systems.[5–7,9]

The deazapurines show a great variety of biological activities, among them, 7-deazapurine nucleoside analogues have demonstrated potent antiviral,[10] and antibacterial activities.[11] Rosemeyer et al.[12] have described a 7-deazaxanthine derivative as an irreversible inhibitor of xanthine oxidase.

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9-Substituted 9-deazaguanines have been described as purine nucleoside phosphorylase (PNP) inhibitors.[13–15] The isosteric replacement of a heterocyclic nitrogen by a carbon unit is a well-established procedure for the study of the interactions of purines with their biological targets.[16,17]

In this study, a series of 9-benzoyl 9-deazaguanine derivatives was screened for XO, and since they are structurally correlated with inosine, the PNPs substrate, their inhibitory specificity was also investigated by screening the compounds for human (Hs) and *Schistosoma mansoni* (Sm) PNPs. From these assays, a 3-nitrobenzoyl-derivative was identified as a potent and specific XO inhibitor. Furthermore, its inhibition constant and mechanism modality were determined.

2. Results and discussion

2.1. Synthesis

Few methods are reported in the literature for the synthesis of pyrrolo[3,2-*d*]pyrimidine (9-deazapurines). One of the strategies involves the formation of the pyrimidine ring onto a preformed 3-aminopyrrole intermediate.[18–20] However, the most generally used approach to pyrrolo[3,2-*d*]pyrimidines has so far involved elaboration of the pyrrole ring onto a preformed pyrimidine bearing reactive functionalities at C-4 and C-5.[21–23]

In this work, we have synthesized 9-benzoyl 9-deazapurines based on the work described by Shih et al.[24] (Scheme 1). Several procedures are reported for the preparation of the starting material, 2-amino-4(1*H*)-pyrimidinone (**1**), using guanidine, ethyl acetoacetate, with or without base, under reflux of ethanol or methanol for several hours.[25,26] We have tested some of the reported conditions and using reflux of ethanol for 18 h, compound **1** was obtained in 81% yield.

Schmink et al.[27] described a microwave (MW) assisted synthesis of 6-methylthiouracil using KOH in ethanol. In an ongoing program toward the development of greener procedures for the preparation of heterocycles,[28,29] we have evaluated different reaction conditions to prepare pyrimidinone **1**, including MW irradiation (Table 1). The best result was obtained without base and in neat conditions under MW irradiation for 10 min at 120 °C affording **1** in 65% yield.

The synthesis of 9-deazapurines was carried out by nitration of **1** furnishing 2-amino-5-nitropyrimidin-4(1*H*)-one (**2**), followed by alkylation using *N*,*N*-dimethylformamide dimethyl acetal furnishing compound **3**.[30] Then, this compound was treated with sodium dithionite in water under reflux for 2 h affording 2-amino-3,5-dihydro-pyrrolo[3,2-*d*]pyrimidin-4-one (**5**) in only 61% yield. We tested this reaction under MW irradiation at 90 °C and after 30 min we obtained a mixture of compound **5** and a by-product, the imidamide **4**,[31] in 38% and 43% yield, respectively. This imidamide was hydrolyzed under basic conditions to furnish the desired product **5**.

Based on this result and following the procedure described by Taylor and Young,[32] treatment of compound **3** with sodium dithionite in a 2:1 mixture of THF/H_2O at rt for 2 h, afforded the imidamide **4** which was then submitted to hydrolysis, using NaOH in water under MW irradiation at 90 °C for 15 min, furnishing compound **5** in 91% yield, after 2 steps. Finally, Friedel–Crafts reaction using 12 different acyl chlorides and trifluoromethane sulfonic acid as catalyst,[24] followed by hydrolysis lead to the 9-benzoyl 9-deazapurine series (**LSPN440–452**), in moderate to good yields (Table 2).

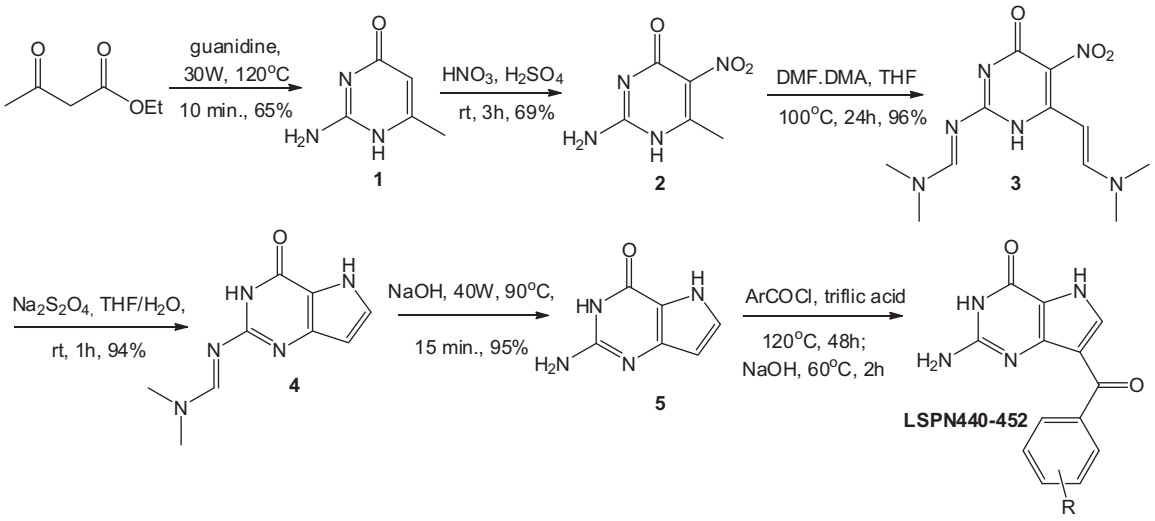
Table 1
Synthesis of 2-amino-4(1*H*)-pyrimidinone (**1**)

Entry	Solvent	Heating	Temperature (°C)	Time (min)	Yield[b] (%)
1	MeOH	Conventional	Reflux	1080	65
2	EtOH	Conventional	Reflux	1080	81
3	MeOH[a]	Conventional	45	480	66
4	—	MW	80	5	27
5	—	MW	100	5	32
6	—	MW	120	5	43
7	—	MW	120	10	65
8	—	MW	120	15	65
9	MeOH	MW	65	15	25
10	MeOH	MW	65	30	38
11	MeOH	MW	65	45	45
12	EtOH	MW	75	15	33
13	EtOH	MW	75	30	55

[a] MeONa was employed as base.
[b] Isolated yield.

2.2. Screening assay

The products of XO and PNP catalyzes (uric acid and hypoxanthine, respectively) were used to determine the enzymatic



Scheme 1. Synthesis of 9-benzoyl 9-deazapurines.

Table 2
Yields obtained in the synthesis of 9-benzoyl 9-deazapurines

Entry	Compound LSPN	R	Yield[a] (%)
1	**440**	H	53
2	**441**	4-Fluoro	64
3	**442**	4-Chloro	68
4	**443**	4-Bromo	65
5	**444**	4-Iodo	56
6	**445**	3-Fluoro	71
7	**446**	3-Chloro	73
8	**447**	2-Chloro	61
9	**448**	3,4-Dichloro	75
10	**449**	3,5-Dichloro	62
11	**450**	2,4,6-Trichloro	52
12	**451**	3-Nitro	68

[a] Isolated yields after purification by column chromatography.

activities of XO-ICERs and PNP-ICERs. Figure 1 shows the enzymatic routes for PNP and XO catalyzes.

Ten out of the twelve compounds synthetized were screened for XO, SmPNP and HsPNP inhibition (Table 2). For that, previously on-flow 2D LC assays were used.[5–7] The screening assay was carried out at high concentrations levels (100 and 200 μM) to select inhibitors that have different potency. The screening was carried in a single concentration level against a control sample. Table 3 furnishes the percentage of inhibition for all compounds. In general, the 9-benzoyl 9-deazapurine derivatives showed none or very weak activity toward the PNPs, being significantly more potent to XO. Due to the higher selectivity index of **LSPN451** toward XO, this compound was selected for kinetics evaluation and mechanistic studies.

The IC_{50} value found for compound **LSPN451** was 65 ± 9.4 nM. To determine the inhibition mechanism, a visual inspection of Lineweaver–Burk (LB) plots, as well as an analysis of the statistical significance in both slope and intercept replots[33] was carried out.

The visual enquiries of LB (Fig. 2) with statistical analysis of slope and *y*-intercept replot data were used to determine the mode of inhibition of compound **LSPN451**. The LB plot showed that K_M and V_{max} decreases curvilinear with increasing inhibitor concentration indicating a non-competitive inhibition with $\alpha < 1$.[34] That was confirmed by statistical analysis of slope and *y*-intercept replot data which showed a significantly non-zero slopes in the

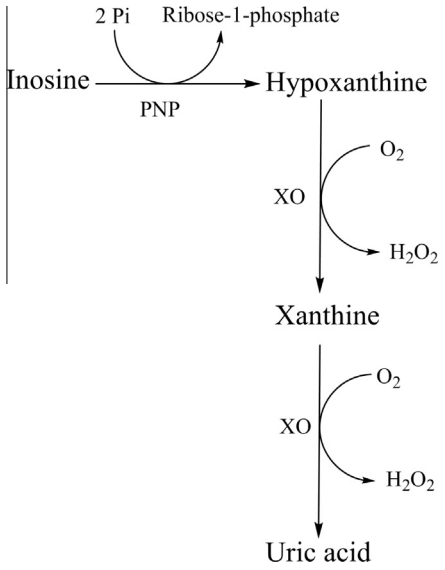


Figure 1. Enzymatic route of purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO).

Table 3
Inhibition selectivity of 9-benzoyl 9-deazapurine derivatives

Compound	Inhibition (%)		
	XO-ICER*	HsPNP-ICER**	SmPNP-ICER**
LSPN440	44.0	0.0	0.0
LSPN441	42.1	0.0	0.0
LSPN442	27.1	7.13	2.52
LSPN443	57.3	19.8	12.8
LSPN444	15.5	0.0	0.0
LSPN446	53.5	0.0	6.39
LSPN447	41.4	0.0	0.0
LSPN450	0.0	0.0	0.0
LSPN451	83.6	25.7	1.50

Inhibition (%) at 100 μM (*) and 200 μM (**). Mean percent inhibition is reported ($n = 2$)**.

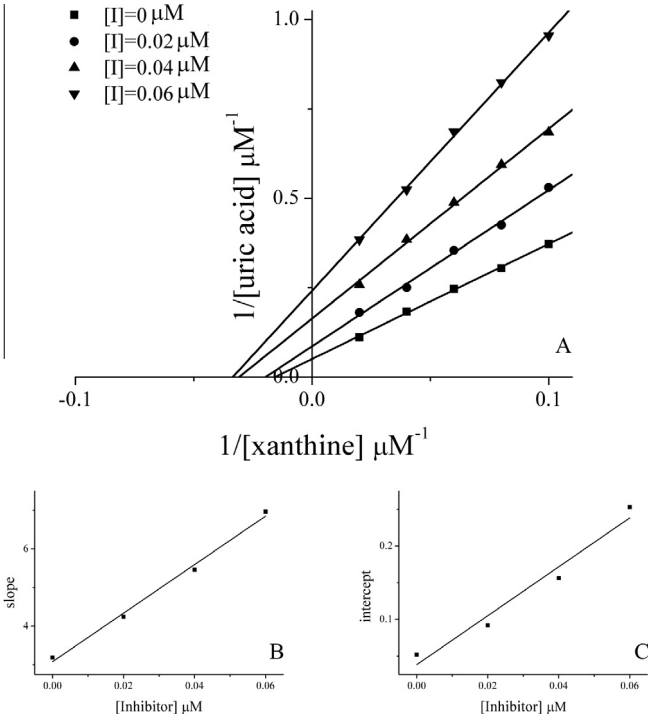


Figure 2. Lineweaver–Burk plot (A) and slope/intercept replots (B and C, respectively) of xanthine oxidase inhibition by compound **LSPN451**. Each point of the LB plot represents the average of duplicate determinations.

regression line for both slope and intercept.[33] These results denote that the inhibitor has higher affinity for the enzyme–substrate complex than for the free enzyme.

Afterward, the inhibition constant was calculated fitting the data to the appropriate nonlinear regression model, which resulted in a K_i = 55.1 ± 9.8 nM with α = 0.29 ± 0.095 that was similar to the obtained IC_{50} value (IC_{50} = 65.2 ± 9.4 nM).

The determined K_i places **LSPN451** as a hit to control uric acid-related health problems. At the same assay conditions the determined K_i value for allopurinol was of only 1.55 μM.[7]

3. Experimental section

3.1. General experimental information

The enzyme xanthine oxidase from bovine milk (E.C. 1.17.3.2) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Hs- and SmPNP expression and purification were conducted as reported elsewhere.[35,36]

Unless otherwise noted, all commercially available reagents were purchased from Sigma–Aldrich Co. Reagents and solvents were purified when necessary according to the usual procedures described in the literature.

For analytical thin-layer chromatography a 0.25 μm film of silica gel containing fluorescent indicator UV254 supported on an aluminum sheet (Sigma–Aldrich) were used. Silica gel (Kieselgel 60, 230–400 mesh, E. Merck) was used for flash column chromatography.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX-400 (400 and 100 MHz, respectively). The IR spectra refer to films and were measured on a Bomem M102 spectrometer.

Gas chromatography was performed in a Shimadzu GC-17A with H_2 as carrier and using a DB-5 column (30 m × 0.25 mm ID, 0.25 μm film thicknesses, J and W Scientific). Melting point was obtained on a MQAPF-301 apparatus. Reactions were carried out in a CEM Discovery® focused microwave oven.

The exact mass measurement was carried out using a Shimadzu Nexera X2 UHPLC system coupled to a Bruker Impact HD QqTOF mass spectrometer (FAPESP PROEM 2014/50244-6). A Betasil® C18 (20 × 2.1 mm, 5 μm) pre-column was used for injection.

3.2. Synthesis of 2-amino-6-methylpyrimidin-4(3*H*)-one (1)[37]

Guanidine (0.2 g, 3.38 mmol) and ethyl acetoacetate (0.45 g, 3.45 mmol) (and eventually 3 mL of solvent) were placed in a glass tube, sealed and irradiated during 20–60 min in a microwave oven, as shown in Table 1. The crude mixture was cooled in an ice bath and a precipitate was observed. The solid was filtered and washed thoroughly with distilled water (5 mL). The dry product was obtained as a white solid. The solvent-free reaction afforded the desired product **1** in 65% yield (274 mg) as a white solid, mp: 296–298 °C (lit. mp >300 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.97 (s, 3H), 5.37–5.39 (m, 1H), 6.50–6.80 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 23.2, 100.2, 155.5, 156.0, 165.1. IR (ν_{max}, film, cm^{-1}): 771, 829, 1018, 1047, 1504, 1666, 2943, 3074, 3332.

3.3. Synthesis of 2-amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (2)[38,39]

In a two-neck round-bottom flask containing precooled sulfuric acid (3 mL) was added 2-amino-4-hydroxy-6-methylpyrimidine (**1**) (100 mg, 0.8 mmol). An effective temperature control is required to the nitration occur properly. Nitric acid (0.2 mL, 3.0 mmol) was added to the solution dropwise maintaining the temperature below 6 °C using an ice bath. The mixture was warmed to room temperature and stirred for 3 h. The reaction mixture was added in cold ethyl ether (10 mL), remaining the temperature below 10 °C. The precipitate was filtered and dissolved in boiling 1 mol/L NaOH solution. Acetic acid was added to the solution for precipitation of the product (pH among 6–8). The solid was filtered under vacuum, washed with water (2 × 1 mL) and dried in vacuum for 8 h. The pyrimidinone **2** was obtained in 69% yield (117 mg) as a pale yellow solid, mp: >300 °C (lit. mp = 300 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.24 (s, 3H), 11.62 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 22.2, 128.5, 155.0, 155.4, 164.4. IR (ν_{max}, film, cm^{-1}): 717, 788, 852, 1093, 1402, 1550, 1658, 1708, 3053, 3498, 3575.

3.4. Synthesis of *N*′-[4-[2-(dimethylamino)ethenyl]-1,6-dihydro-5-nitro-6-oxo-2-pyrimidinyl]-*N*,*N*-dimethylmethanimidamide (3)[22]

In a 100 mL two-neck round-bottom flask under N_2 atmosphere and molecular sieve 3 Å was added **2** (2.0 g, 11.7 mmol) in dry DMF (25 mL) and DMF dimethyl acetal (7.5 mL, 70.0 mmol). The mixture was stirred at 100 °C for 24 h and then cooled. The mixture was dried under vacuum. Acetone (15 mL) was added and the formed precipitate was filtered and washed with acetone (10 mL) furnishing **3** in 96% yield (3.16 g) as an orange solid, mp: 276–278 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.58 (s, 1H), 7.88 (d, J = 12.2 Hz, 1H), 5.60 (d, J = 12.2 Hz, 1H), 3.24 (s, 3H), 3.14 (s, 3H), 2.80–3.10 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 35.2, 41.5, 89.7, 124.4, 151.7, 156.2, 156.4, 157.5, 158.4. IR (ν_{max}, film, cm^{-1}): 3429, 2929, 1666, 1608, 1542, 1477, 1388, 865, 752, 740.

3.5. Synthesis of 2-amino-3,5-dihydro-pyrrolo[3,2-*d*]pyrimidin-4-one (5)[28]

In a 500 mL round-bottom flask were combined **3** (4.6 g, 16.4 mmol), sodium dithionite (12.0 g, 69 mmol) and THF/H_2O (2:1, 300 mL). The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure, then H_2O (50 mL) was added and the solid formed was filtered and washed with water (2 × 100 mL). The product was purified by silica gel chromatography using 2% MeOH in CH_2Cl_2 as eluent. Compound **4** was obtained in 94% yield (3.16 g) as a white solid. ^{1}H NMR (200 MHz, DMSO-d_6) δ (ppm): 11.58 (s, 1H), 8.51 (s, 1H), 7.19 (t, J = 5.9 Hz, 1H), 6.09 (t, J = 4.7 Hz, 1H), 3.13 (s, 3H), 3.03 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 39.7, 41.3, 103.3, 115.6, 129.2, 145.2, 154.4, 156.7, 157.3. IR (ν_{max}, film, cm^{-1}): 3431, 3166, 1685, 1620, 1492, 1400, 1336, 1108, 1093, 784.

In the sequence, in a microwave tube was added **4** (200 mg, 0.97 mmol) and NaOH (195.0 mg, 4.84 mmol) in H_2O (5 mL). The set conditions were 95 °C for 15 min with an average power of 40 W. This procedure was repeated four times and then the crude products were mixed. The solvent was evaporated under vacuum and then water (5 mL) was added. The mixture was acidified to pH 5.0 with glacial acetic acid. The precipitate was filtered and then recrystallized in ethanol/H_2O (3:1) affording the desired product **5** in 96% yield (701.0 mg) as a white solid, mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.43 (d, J = 5.9 Hz, 1H), 6.44 (s, 2H), 5.87 (d, J = 4.9 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 100.1, 112.0, 127.4, 144.8, 151.2, 153.0. IR (ν_{max}, film, cm^{-1}): 3450, 3207, 3134, 1647.

3.6. General procedure for 9-benzoyl 9-deazapurines LSPN440–451

A mixture of 9-deazapurine **5** (150.0 mg, 1.0 mmol) and benzoyl chloride (2.2 equiv) in trifluoromethanesulfonic acid (3.45 g, 23 mmol) was stirred at 80–120 °C for 48 h. After the mixture was cooled and H_2O (15 mL) was added. Then the reaction was neutralized with NaOH 1.0 mol/L, the volume adjusted to approximately 35 mL by adding H_2O and then 60 equiv of NaOH were added. The reaction was stirred for 2.5 h at 60 °C. The mixture was neutralized with glacial acetic acid and the formed solid was filtered under vacuum and washed several times with dichloromethane (10 mL), ethyl acetate (10 mL), acetone (5 mL) and methanol (5 mL). The precipitate purity was monitored by CCD and depending on the product, further purification was necessary by recrystallization in methanol or flash chromatography using CH_2Cl_2/MeOH (4:1) as eluent.

3.6.1. 2-Amino-3*H*,5*H*-7-benzoylpyrrolo[3,2-*d*]pyrimidin-4-one (LSPN440)[24]

White solid (135 mg), mp: degraded at 280 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.72–7.68 (m, 2H), 7.54–7.44 (m, 3H), 7.41 (s, 1H), 6.60 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 188.5, 156.1, 152.6, 146.5, 142.2, 141.6, 130.4, 128.6,

128.0, 114.2, 100.1. IR (ν_{max}, film, cm^{-1}): 3427, 3114, 3083, 1703, 1525, 1483, 1411, 798, 769.

3.6.2. 2-Amino-3*H*,5*H*-7-(4-fluorbenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN441)

White solid (174 mg), mp: degraded at 294 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.78–7.74 (m, 2H), 7.41 (s, 1H), 7.27 (t, J = 17.7 Hz, 2H), 6.65 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 186.9, 164.5, 162.0, 156.4, 152.4, 146.6, 142.9, 138.2, 131.1, 121.3, 114.9, 114.7, 114.1. IR (ν_{max}, film, cm^{-1}): 3406, 3083, 1703, 1525, 1485, 1415, 1228, 1153, 790, 775.

3.6.3. 2-Amino-3*H*,5*H*-7-(4-chlorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN442)

White solid (196 mg), mp: degraded at 280 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.70 (d, J = 8.3 Hz, 2H), 7.53 (d, J = 8.3 Hz, 2H), 7.36 (s, 1H), 6.57 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 186.6, 156.7, 152.4, 147.1, 144.3, 140.7, 139.2, 134.6, 130.9, 130.4, 127.9, 127.1, 113.9. IR (ν_{max}, film, cm^{-1}): 3407, 3080, 1699, 1527, 1481, 1413, 1282, 1191, 777, 761. HRMS: calc. for $C_{13}H_{10}ClN_4O_2$: 289.70; exp. 289.0493.

3.6.4. 2-Amino-3*H*,5*H*-7-(4-bromobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN443)

White solid (216 mg), mp: degraded at 288 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.86 (d, J = 8.3 Hz, 2H), 7.52–7.58 (m, 3H), 6.70 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 187.7, 155.0, 152.8, 145.2, 139.8, 136.9, 135.9, 131.3, 130.8, 114.1, 98.6. IR (ν_{max}, film, cm^{-1}): 3425, 3116, 3083, 1703, 1525, 1413, 1147, 775.

3.6.5. 2-Amino-3*H*,5*H*-7-(4-iodobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN444)

White solid (213 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.82 (d, J = 8.3 Hz, 2H), 7.46 (d, J = 8.3 Hz, 2H), 7.29 (s, 1H), 6.51 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 186.7, 157.0, 152.3, 147.5, 145.5, 141.7, 136.7, 130.5, 123.8, 113.9, 96.9. IR (ν_{max}, film, cm^{-1}): 3423, 3116, 3080, 1703, 1525, 1411, 1147, 775, 754.

3.6.6. 2-Amino-3*H*,5*H*-7-(3-fluorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN445)

White solid (193 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.53–7.50 (m, 2H), 7.38–7.30 (m, 3H), 6.52 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 186.1, 162.9, 160.5, 157.0, 152.4, 144.7, 129.9, 124.5, 116.7, 116.5, 114.9, 114.7, 113.8. IR (ν_{max}, film, cm^{-1}): 3427, 3083, 1701, 1411, 1238, 786, 723.

3.6.7. 2-Amino-3*H*,5*H*-7-(3-chlorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN446)

White solid (216 mg), mp: degraded at 270 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.43 (s, 1H), 7.73–7.71 (m, 2H), 7.63–7.60 (m, 2H), 7.50 (t, J = 8.0 Hz, 1H), 6.65 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 187.2, 153.9, 152.9, 144.0, 141.5, 133.7, 132.9, 131.3, 130.1, 128.5, 127.8, 114.4, 113.1. IR (ν_{max}, film, cm^{-1}): 3431, 3087, 1701, 1529, 1409, 1211, 1149, 773, 738.

3.6.8. 2-Amino-3*H*,5*H*-7-(2-chlorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN447)

White solid (176 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.54–7.42 (m, 4H), 7.25 (s, 1H), 6.75 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 187.1, 153.9, 153.4, 144.3, 140.3, 135.0, 130.6, 129.6, 129.5, 128.6, 126.9, 115.1, 113.7. IR (ν_{max}, film, cm^{-1}): 3448, 3130, 3091, 1691, 1529, 1417, 1149, 1068, 769, 717.

3.6.9. 2-Amino-3*H*,5*H*-7-(3,4-dichlorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN448)[24]

White solid (243 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.79 (d, J = 2.0 Hz, 1H), 7.69 (d, J = 8.3 Hz, 1H), 7.61 (dd, J = 2.0 and 8.7 Hz, 1H), 7.13 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 188.6, 154.9, 151.4, 140.1, 137.8, 135.8, 135.0, 133.5, 130.1, 126.4, 113.2, 99.1. IR (ν_{max}, film, cm^{-1}): 3417, 3126, 3095, 1697, 1529, 1487, 1463, 1211, 1189, 773, 761.

3.6.10. 2-Amino-3*H*,5*H*-7-(3,5-dichlorobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN449)[24]

White solid (200 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.49 (s, 1H), 7.68 (s, 1H), 7.50–7.43 (m, 3H), 6.68 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 190.2, 153.4, 150.5, 138.0, 135.2, 134.3, 132.5, 131.9, 130.0, 126.8, 125.9, 102.5, 96.3. IR (ν_{max}, film, cm^{-1}): 3431, 3087, 1701, 1529, 1487, 1436, 1413, 1168, 1120, 771.

3.6.11. 2-Amino-3*H*,5*H*-7-(2,4,6-trichloro)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN450)

White solid (218 mg), mp: degraded at 296 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 6.29 (s, 2H), 6.84 (s, 1H), 7.64 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 177.6, 161.5, 159.6, 152.7, 142.5, 140.2, 132.3 (2C), 132.2 (2C), 120.9, 113.1. IR (ν_{max}, film, cm^{-1}): 3481, 3230, 3080, 1670, 1583, 1514, 1415, 1201, 781.

3.6.12. 2-Amino-3*H*,5*H*-7-(3-nitrobenzoyl)pyrrolo[3,2-*d*] pyrimidin-4-one (LSPN451)[24]

White solid (203 mg), mp: degraded at 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.47 (s, 1H), 8.35 (d, J = 9.3 Hz, 1H), 8.13 (d, J = 7.3 Hz, 1H), 7.76 (t, J = 15.8 Hz, 1H), 7.38 (s, 1H), 6.37 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 184.9, 156.3, 153.6, 148.6, 137.3, 136.3, 135.3, 132.8, 128.0, 126.4, 124.7, 107.6, 105.7. IR (ν_{max}, film, cm^{-1}): 3485, 3323, 3195, 3085, 1706, 1529, 1433, 1215, 781, 723. HRMS: calc. for $C_{13}H_{10}N_5O_4$: 300.07; exp. 300.0729.

3.7. Screening and inhibition studies

The protocols to prepare XO and PNP-ICERs, as well as the validated LC 2D methods for quantifying the enzymatic reactions products are described in previously works.[3–5]

Ten 9-benzoyl 9-deazaguanine derivatives were screened with XO-ICER. Stock solutions of each compound were prepared in methanol/H_2O (1:1, v/v) at 2 mM. The compounds were evaluated at 100 μM using XO-ICER and 200 μM for PNP-ICERs.

The assay solutions for XO-ICER were prepared with the addition of 100 μL of xanthine (200 μM), 80 μL water and 20 μL of methanol/H_2O (1:1, v/v) solution, maintaining methanol concentration fixed in all samples. Aliquots of 10 μL were injected in the chromatographic system[3,5] and, the enzymatic activity evaluated by the quantification of uric acid produced by XO-ICERs at 294 nm. For each tested compound, a control sample was prepared using a solution of methanol/H_2O (1:1, v/v) to substitute the compound solutions, measuring the impact of methanol in the enzyme activity.

To investigate the inhibition specificity, the same library was screened for Sm- and HsPNP. Inhibitory activity of these analogues against SmPNP was evaluated at 200 μM, by injecting 15 μL aliquots of a sample containing Ino 250 μM, phosphate buffer (pH 7.4) 400 μM, and 200 μM of the assayed compound. The screening for HsPNPs was carried out at 200 μM, by injecting 20 μL of a sample containing 5 mM phosphate buffer pH 7.0 and 382.5 μM Ino.

The percentage of inhibitions were calculated by the following expression: % $I = 100 - (Ci/C0 \times 100)$, where Ci is the enzymatic

product (Hypo or uric acid) concentration obtained in the presence of the tested compounds, and C0 is the enzymatic product concentration calculated in the absence of the evaluated compounds.

An inhibition curve was constructed for **LSPN451** (0, 0.02, 0.05, 0.10, 0.25, 0.50, 2.50, 3.50, 4.00, 6.00, 8.00 and 10.00 μM) at fixed concentration of substrate (25 μM) by plotting the percentage of inhibition versus the inhibitor concentration and, the IC_{50} value was, thus, obtained. The modality of inhibition was determined by visual inspection of Lineweaver–Burk plot and statistical analysis of slope and *y*-intercept replot data. The slopes of replot were analyzed by GraphPad Prism software to determine its significantly nonzero slopes in the regression line using *F*-test feature.[31]

Then, based in the obtained inhibition mechanism, the appropriate nonlinear regression model was selected in the GraphPad Prism software to obtain K_i and α values.

4. Conclusions

A series of new 9-benzoyl 9-deazaguanine derivatives were efficiently synthesized and ten compounds were investigated for their inhibition against XO and Sm- and HsPNPs. The 2D LC assay system, with the XO-ICER and PNP-ICERs at the first dimension, has once again showed to be a valuable tool, not only for selectively identifying the inhibitors, but also for mechanistically characterizing them. **LSPN451** emerged as a potent selective XO noncompetitive inhibitor with higher affinity for the enzyme–xanthine complex.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.12.006.

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