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Synthesis of Purine and 7-Deazapurine Nucleoside Analogues of 6-*N*-(4-Nitrobenzyl)adenosine; Inhibition of Nucleoside Transport and Proliferation of Cancer Cells

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Human equilibrative nucleoside transporter 1 (hENT1) is a prototypical nucleoside transporter protein ubiquitously expressed on the cell surface of almost all human tissue. Given the role of hENT1 in the transport of nucleoside drugs, an important class of therapeutics in the treatment of various cancers and viral infections, efforts have been made to better understand the mechanisms by which hENT1 modulates nucleoside transport. To that end, we report here the design and synthesis of novel tool compounds for the further study of hENT1. The 7-deazapurine nucleoside antibiotic tubercidin was converted into its 4-*N*-benzyl and 4-*N*-(4-nitrobenzyl) derivatives by alkylation at N3 followed by a Dimroth rearrangement to the 4-*N*-isomer or by fluoro-diazotization followed by S_NAr displacement of the 4-fluoro group by a benzylamine. The 4-*N*-(4-nitrobenzyl) derivatives of sangivamycin and toyocamycin antibiotics were prepared by the alkylation approach. Cross-membrane transport of labeled uridine by hENT1 was inhibited to a weaker extent by the 4-nitrobenzylated tubercidin and sangivamycin analogues than was observed with 6-*N*-(4-nitrobenzyl)adenosine. Type-specific inhibition of cancer cell proliferation was observed at micromolar concentrations with the 4-*N*-(4-nitrobenzyl) derivatives of sangivamycin and toyocamycin, and also with 4-*N*-benzyltubercidin. Treatment of 2′,3′,5′-*O*-acetyladenosine with aryl isocyanates gave the 6-ureido derivatives but none of them exhibited inhibitory activity against cancer cell proliferation or hENT1.

Introduction

Analogues and derivatives of 7-deazapurine nucleoside antibiotics have been synthesized and subjected to extensive biological testing.[1] A sizeable number of active nucleoside compounds with such a pyrrolo[2,3-*d*]pyrimidine base has been prepared by chemical modifications of the parent antibiotics as well as by coupling base derivatives with protected sugars.[2] Noteworthy examples of such molecules include: 5-iodotubercidin,[3] an up-field activator of the p53 pathway; sangivamycin analogues, such as 6-hydrazinosangivamycin[4] and xylocidine,[5] in vitro down-field inhibitors of protein kinase C and cyclin-dependent kinases in cancer cell lines; a methyl-substituted tubercidin,[6] which acts against the replication of polio and dengue viruses; the anti-herpes simplex virus agent xylotubercidin;[7] substituted toyocamycin analogues[8] and 2′-β-*C*-methyl derivative of toyocamycin,[9] sangivamycin,[9] and tubercidin[10] that have activity against the hepatitis C virus; 2′-deoxy-2′-fluoroarabinotubercidin[11] and 2-amino-2′-deoxy-2′-fluoroarabinotubercidin,[12] which exhibit antiviral activity; 4*N*,5-diaryltubercidin derivatives, which act as adenosine kinase inhibitors;[2l,13] and tubercidin derivatives, such as 4-(het)aryl,[14] 5-(het)aryl[15] and some 4-substituted-5-(het)aryl[16] compounds with nanomolar cytostatic activity against several cancer cell lines.

Various methods to access 4-*N*-substituted-7-deazapurine derivatives have been reported;[8,16–18] however, direct alkylation of exocyclic amino groups on 7-deazapurine nucleosides has thus far received much less attention.[2b] Thus, treatment of tubercidin with methyl iodide followed by sodium hydroxide produced 4-*N*-methyltubercidin[2b] (6-*N*-methyl-7-deazaadenosine) via a Dimroth rearrangement. Several other 4-*N*-substituted tubercidin derivatives have previously been prepared by S_NAr displacement of chloride from the 4-chloro analogue[2b,16] of tubercidin or by displacement of 1,2,4-triazole from 4-*N*-(1,2,4-triazol-4-yl) intermediates.[17] Such 4-*N*-substituted toyocamycin derivatives also were prepared from the corresponding 4-chloro compounds.[8] The 7-chloro derivative of the *C*-nucleoside antibiotic formycin (3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine) was prepared and then converted into 7-*N*-benzylformycin.[18]

Purine and pyrimidine nucleosides and their derivatives play crucial roles in human physiology and pharmacology.[19] These "salvage" metabolites can be converted into nucleotides, which in turn serve as the energy-rich currency of intermediary metabolism and as precursors of nucleic acid biosynthesis. Ad-

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enosine functions as a key signaling molecule and modulates diverse physiological processes through interactions with cell surface P1 purinergic receptors.

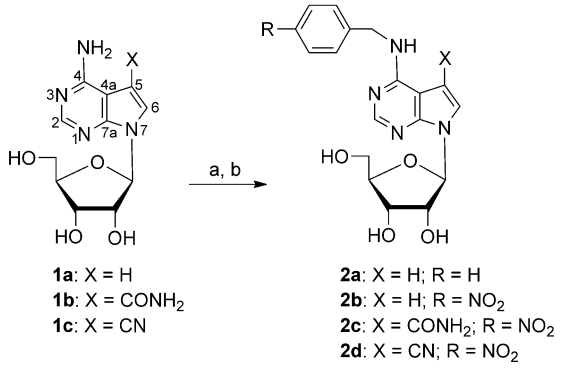
Many types of cancer and viral infections are treated with nucleoside drugs,[20] but drug availabilities within cells and in the extracellular environment are determined primarily by specific nucleoside transporter (NT) proteins that facilitate their movement across plasma membranes and some organellar membranes. Because of the hydrophilic nature of nucleosides, NTs play key roles in the physiology, pathophysiology, and therapeutic actions of many nucleoside drugs.[19] The human equilibrative nucleoside transporter 1 (hENT1) is the prototypical NT present in the outer plasma membrane and some intracellular membranes of most, if not all, human cells.[21]

Nitrobenzylmercaptopurine ribonucleoside (NBMPR) and structurally related hENT1 probes such as 6-*N*-(4-nitrobenzyl)adenosine[22] and 5′-*S*-{2-(6-[3-(fluorescein-5-yl)thioureido-1-yl]-hexanamido)}ethyl-6-*N*-(4-nitrobenzyl)-5′-thioadenosine (FITC-SAHENTA)[23] bind with nanomolar affinity within, or closely associated with, the outward-facing permeant-binding site of hENT1. Development of such probes has greatly aided investigations on the structure, function, and cell-surface abundances of hENT1.[19,23,24] We now report the synthesis of analogues of these transport inhibitors with purine and 7-deazapurine bases that exhibit pronounced cytotoxicity in several cancer cell lines as well as moderate inhibition of hENT1 transport activity.

Results and Discussion

Chemistry

The *N*-benzylated 7-deazaadenosine analogues (**2a**–**d**) (Scheme 1) were prepared by two procedures. One involved alkylation of the 7-deazaadenosine antibiotics (**1a**–**c**) at N3 with a benzyl bromide followed by a base-promoted Dimroth rearrangement.[18,25] The second route employed diazotization–fluorodediazoniation followed by S_NAr displacement of fluoride with a benzyl amine.[26] Alkylation of adenosine occurs at N1, and the resulting cation undergoes rearrangement in basic solution to give the N6-alkylated product.[27] Alkylation of tubercidin (**1a**) with benzyl bromide in DMF occurred at N3 (same as ring nitrogen N1 in adenosine), and treatment of the 3-benzyl intermediate with dimethylamine (2 M in THF) in methanol resulted in rearrangement to give 4-*N*-benzyltubercidin (**2a**; 67%).



1a: X = H
1b: X = $CONH_2$
1c: X = CN

2a: X = H; R = H
2b: X = H; R = NO_2
2c: X = $CONH_2$; R = NO_2
2d: X = CN; R = NO_2

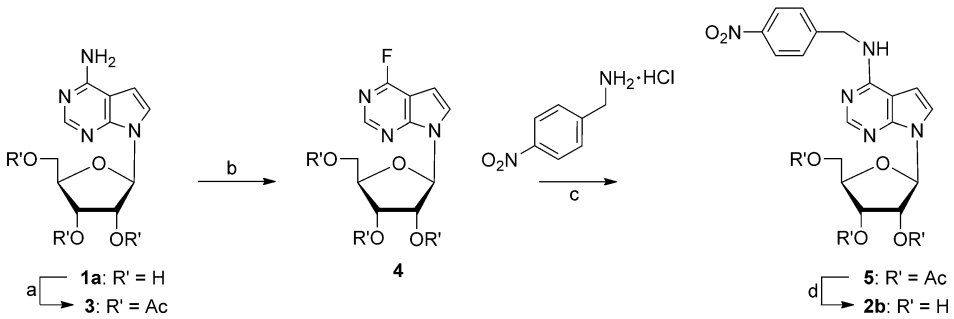
Scheme 1. Synthesis of 4-*N*-benzylated nucleosides by alkylation. *Reagents and conditions*: a) $ArCH_2Br$, DMF, 40–80 °C, 24–63 h; b) Me_2NH (2 M in THF), MeOH, reflux, 20–32 h. Yield: 45–67%.

Because the 6-*N*-(4-nitrobenzyl) derivatives of adenosine and other adenine nucleosides are more potent inhibitors of nucleoside transport,[22] we also synthesized 4-nitrobenzyl derivatives of the 7-deaza antibiotics. Alkylation of **1a** with 4-nitrobenzyl bromide followed by treatment with dimethylamine (2 M in THF) in methanol produced 4-*N*-(4-nitrobenzyl)tubercidin (**2b**; 56%). Analogous treatment of sangivamycin (**1b**) with 4-nitrobenzyl bromide and then dimethylamine (2 M in THF) in methanol gave 4-*N*-(4-nitrobenzyl)sangivamycin (**2c**; 45%).

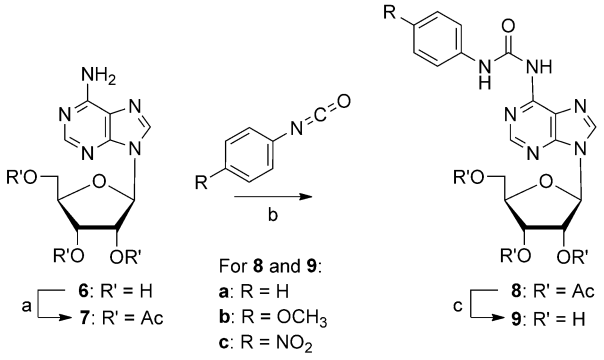
Treatment of toyocamycin (**1c**) with 4-nitrobenzyl bromide unexpectedly gave 4-*N*-(4-nitrobenzyl)toyocamycin (**2d**) directly. Formation of an intermediate alkylated at N3 apparently did not occur; TLC analysis of the reaction mixture showed only a more rapidly migrating material corresponding to **2d** without indication of a more polar (cationic) product. Therefore, successive treatment with dimethylamine was not required. Although direct alkylation on the exocyclic amino group of adenine and guanine with quinone methides is known,[28] the observed direct alkylation on the exocyclic amine group of toyocamycin with 4-nitrobenzyl bromide has not been previously reported. This might have resulted from alteration of the nucleophilicity of the endocyclic N3 of toyocamycin relative to that of the exocyclic 4-amino nitrogen by the electron-withdrawing pull of the cyano group at C5. A possible accompanying stereoelectronic effect might result from interference of the cylindrical cyano group with a synperiplanar orientation of the exocyclic amino group relative to the planar heterocyclic ring that would diminish lone-pair donation from the amino nitrogen toward N3 of that amidine system.[29]

Alternatively, tubercidin (**1a**) was acetylated and then subjected to diazotive-fluorodeamination[23,30] followed by S_NAr displacement of fluoride with benzylamines (Scheme 2).[23,26] Thus, 2′,3′,5′-tri-*O*-acetyltubercidin (**3**) was treated with sodium nitrate in freshly prepared hydrogen fluoride pyridine (~55%) at −10 °C[31] for 15 minutes to yield protected 4-fluorotubercidin **4** (82%). The concentration of hydrogen fluoride pyridine as well as the temperature are crucial.[31b] Treatment of **4** with 4-nitrobenzylamine hydrochloride in methanol and in the presence of triethylamine[23] gave 2′,3′,5′-tri-*O*-acetyl-4-*N*-(4-nitrobenzyl)tubercidin (**5**; 47%), which was deacetylated (ammonia in methanol) to give **2b** (85%).

Some 2′,3′-bis-*O*-silylated adenosine derivatives with ureido substituents at the 5′- and 6-positions exhibit antiproliferative activity.[32] We prepared the 6-*N*-[*N*-arylcarbamoyl]adenosine derivatives (**9a**–**c**; Scheme 3) with phenyl, 4-methoxyphenyl, and 4-nitrophenyl moieties to probe for binding to hENT1 and inhibition of nucleoside transport as observed with 6-*N*-(4-nitrobenzyl)adenosine.[22] The 2′,3′,5′-tri-*O*-acetyl derivative (**7**) of adenosine (**6**) was treated with phenyl isocyanate to give **8a**. Deacetylation gave 6-*N*-[*N*-phenylcarbamoyl]adenosine (**9a**),



Scheme 2. S_NAr-based synthesis of 4-*N*-(4-nitrobenzyl)tubercidin (**2b**). *Reagents and conditions*: a) Ac_2O, Py, 0 °C→RT, 21 h, 86%; b) Py·HF (55% HF), $NaNO_2$, −10 °C, 0.25 h, 82%; c) Et_3N, MeOH, RT, 5 h, 47%; d) saturated NH_3 in MeOH, RT, 20 h, 85%.



Scheme 3. Synthesis of 6-ureidoadenosine analogues. *Reagents and conditions*: a) Ac_2O, Py, 0 °C→RT, 24 h, 90%; b) CH_2Cl_2, RT, 24–68 h, 80–96%; c) saturated NH_3 in MeOH, RT, 2 h, 65–85%.

and the analogous sequential treatment of **7** with the respective isocyanates produced the 4-methoxyphenyl and 4-nitrophenyl analogues **9b** and **9c**.

Nucleoside transport

Inhibition of the initial rate of [^{3}H]-uridine (20 μM) uptake by recombinant hENT1 produced in the *Xenopus* oocyte heterologous expression system was determined as described previously.[21] Minimal inhibition (<25%) of labelled uridine uptake was observed at a concentration of 100 μM for each of the 6-ureidoadenosine compounds **9a**, **9b**, and **9c**. The 4-*N*-benzyltubercidin (**2a**) and 4-*N*-(4-nitrobenzyl)toyocamycin (**2d**) analogues also exhibited weak inhibition (<45%) but both 4-*N*-(4-nitrobenzyl)tubercidin (**2b**) and 4-*N*-(4-nitrobenzyl)sangivamycin (**2c**) fully inhibited labelled uridine uptake at that concentration. Dose–response evaluations indicated that **2c** (IC_{50} = 123 ± 31 nM) was a better inhibitor of uridine transport by hENT1 than **2b** (IC_{50} > 1000 nM). However, the 7-deaza analogue **2c** was a weaker inhibitor of hENT1-mediated transport than 6-*N*-(4-nitrobenzyl)adenosine, NBMPR, and other derivatives[22] containing a nitrogen atom at the 7-position in the adenine ring.

Inhibition of cell proliferation

Inhibition of the proliferation of murine leukemia (L1210) cells and human T-lymphocyte (CEM), cervix carcinoma (HeLa), prostate adenocarcinoma (PC-3), and kidney carcinoma (Caki-1) cells by the adenine and 7-deazaadenine nucleoside analogues was evaluated. No pronounced inhibitory effect was observed with the 6-ureido compounds **9a**, **9b**, and **9c** in any of the tumor cell lines (Table 1). Marked inhibition of the proliferation of PC-3 cells (IC_{50}: 0.92 μM) and HeLa cells (IC_{50}: 7.4 μM) by 4-*N*-benzyltubercidin (**2a**) was observed but no significant activity was found with its nitrobenzyl analogue **2b** (IC_{50}: >50 μM). The 4-*N*-(4-nitrobenzyl)sangivamycin (**2c**) and 4-*N*-(4-nitrobenzyl)toyocamycin (**2d**) analogues inhibited proliferation of L1210, HeLa, and PC-3 cells, with IC_{50} values ranging from approximately 0.9–9.4 μM with **2c** showing more potent effects (IC_{50}: 0.92–3.4 μM). It is intriguing that such striking differences in cytostatic activity were dependent on the nature of the tumor cell lines. HeLa and PC-3 tumor cells were highly susceptible to the cytostatic activity of **2a**, **2c**, and **2d**, whereas the L1210 cells were sensitive only to **2c** and **2d**, and the CEM and Caki-1 cells were weakly sensitive to the antiproliferative effects of any of compounds **2a**–**2d**.

Table 1. Inhibitory effects on the proliferation of cells in culture.

Compd	IC_{50} [μM][a]				
	L1210	CEM	HeLa	PC-3	Caki-1
2a	172 ± 47	182 ± 20	7.4 ± 2.2	0.92 ± 0.67	116 ± 23
2b	125 ± 28	≥250	143 ± 4	76 ± 10	132 ± 30
2c	0.92 ± 0.04	115 ± 28	1.8 ± 0.2	3.4 ± 0.9	116 ± 23
2d	5.5 ± 1.5	109 ± 16	9.4 ± 3.0	5.6 ± 3.3	98 ± 10
9a	>250	221 ± 0	201 ± 44	115 ± 20	150 ± 33
9b	>250	>250	161 ± 10	110 ± 31	≥250
9c	>250	>250	>250	45 ± 26	>250

[a] Half-maximal inhibitory concentration (IC_{50}); data represent the mean ± SD of at least n = 2–3 independent experiments. Cell lines: murine leukemia (L1210); human CD4+ T-lymphocytes (CEM); human cervix carcinoma (HeLa); human prostate adenocarcinoma (PC-3); human kidney carcinoma (Caki-1).

Conclusions

Alkylation at N3 of tubercidin with 4-nitrobenzyl bromide and a Dimroth rearrangement gave 4-*N*-(4-nitrobenzyl)tubercidin, which also was prepared by fluoro-diazotization of tubercidin and S_NAr displacement of the 4-fluoro group by 4-nitrobenzylamine. The alkylation–rearrangement sequence was employed to convert sangivamycin (5-carboxamidotubercidin antibiotic)

into its 4-*N*-(4-nitrobenzyl) derivative. However, no evidence of formation of an initial N3-alkylated intermediate was observed upon treatment of toyocamycin (5-cyanotubercidin antibiotic) with 4-nitrobenzyl bromide. Alkylation occurred directly on the amino group to give 4-*N*-(4-nitrobenzyl)toyocamycin. The 4-*N*-(4-nitrobenzyl) derivatives of tubercidin and sangivamycin inhibited cross-membrane transport of labelled uridine by hENT1 at IC_{50} values of >1 μM and ~120 nM, respectively. Inhibition of the proliferation of L1210, HeLa, and PC-3 tumor cells in culture was observed with 4-*N*-benzyltubercidin and the 4-*N*-(4-nitrobenzyl) derivatives of sangivamycin and toyocamycin at IC_{50} values of 0.92–9.4 μM.

Experimental Section

General: Reagent-grade chemicals were used, and solvents were dried by heating at reflux over CaH_2 under N_2 unless otherwise specified. Reactions were performed under an atmosphere of N_2. Reaction progress was monitored by thin-layer chromatography (TLC) on Merck Kieselgel 60-F_{254} sheets with product detection by 254 nm light. Products were purified by column chromatography using Merck Kiselgel 60 (230–400 mesh) or by automated flash chromatography using a CombiFlash system. Purity of the synthesized compounds was determined to be $\geq$95% by elemental analysis (C, H, N) and/or high-performance liquid chromatography (HPLC).

^{1}H (400 MHz), ^{13}C (100.6 MHz), and ^{19}F (376 MHz) NMR spectra were recorded at RT in solutions of $CDCl_3$ or [D_6]DMSO. Chemical shifts (δ) are reported in parts per million (ppm) referenced to the residual solvent peak, and coupling constants (*J*) are given in Hertz (Hz). Multiplicity is reported using standard abbreviations: singlet (s); doublet (d); triplet (t); multiplet (m); broad (br); inverted commas indicate observed multiplicity. UV spectra were recorded with a Varian Cary 100 Bio UV-visible spectrophotometer.

4-Benzylamino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (2a): BnBr (345 μL, 496 mg, 2.9 mmol) was added to a stirred solution of tubercidin (**1a**; 266 mg, 1 mmol) in dried DMF (5 mL). After stirring for 48 h at 40 °C, TLC showed almost complete conversion to a more polar product. The volatiles were evaporated under vacuum (<40 °C) to ~1 mL, and the resulting syrup was added dropwise to dried acetone (30 mL) with vigorous stirring. Et_2O (60 mL) was added to the resultant suspension, which was then chilled for 20 min at 0 °C. The precipitate was isolated by vacuum filtration. The hygroscopic solid was quickly redissolved in MeOH (10 mL), and Me_2NH in THF (2 M, 8 mL) was added. The resulting solution was heated at reflux (65 °C, oil bath) with stirring for 20 h. TLC showed ~80% conversion to a less polar product, and volatiles were removed in vacuo with coevaporation using MeOH (×2). The residue was redissolved in warm MeOH (10 mL), H_2O (60 mL) was added, and the solution was extracted with EtOAc (5×20 mL). The combined organic phase was dried (Na_2SO_4), filtered, and concentrated in vacuo, with coevaporation using EtOH (×2). Purification by flash chromatography (100%, EtOAc) gave **2a** as a colorless oil (238 mg, 67%): UV (MeOH): λ_{max} = 276 nm, λ_{min} = 244 nm; ^{1}H NMR ([D_6]DMSO): δ = 8.09–8.13 (m, 2H, NH, H2), 7.38 (d, 1H, *J* = 3.7 Hz, H6), 7.29–7.35 (m, 4H, Ph), 7.22–7.25 (m, 1H, Ph), 6.67 (d, 1H, *J* = 3.4 Hz, H5), 6.01 (d, 1H, *J* = 5.9 Hz, H1′), 5.27–5.32 (m, 2H, 2′-OH, 5′-OH), 5.12 (d, 1H, *J* = 4.3 Hz, 3′-OH), 4.70–4.78 (m, 2H, CH_2), 4.44 (q, 1H, *J* = 5.7 Hz, H2′), 4.09 ("q", 1H, *J* = 4.1 Hz, H3′), 3.90 (q, 1H, *J* = 3.4 Hz, H4′), 3.60–3.65 (m, 1H, H5′), 3.50–3.56 ppm (m, 1H, H5″); ^{13}C NMR ([D_6]DMSO): δ = 156.1, 151.4, 149.5, 140.1, 128.2 (Ph), 127.1 (Ph), 126.6, 122.3 (C6), 103.4, 99.2 (C5), 87.6 (C1′), 85.1 (C4′), 73.7 (C2′), 70.7 (C3′), 61.8 (C5′), 43.1 ppm (CH_2); MS (ESI): *m/z* 357 [*M*+H]$^+$ (100%); HRMS (ESI): *m/z* [*M*+H]$^+$ calcd for $C_{18}H_{29}N_4O_4$: 357.1563; found: 357.1581.

4-(4-Nitrobenzylamino)-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (2b)

Method A. Tubercidin (**1a**; 266 mg, 1.0 mmol) was treated with 4-nitrobenzyl bromide (648 mg, 3.0 mmol) at 80 °C for 24 h as described above for **2a**. The resultant solid was redissolved in MeOH (15 mL), and the solution was treated with Me_2NH in THF (2 M, 8 mL). The reaction mixture was heated at reflux (65 °C, oil bath) with stirring for 20 h. TLC showed ~85% conversion to a less polar product, which was isolated and purified as described for **2a** to give **2b** as a yellow oil (244 mg, 56%): UV (MeOH): λ_{max} = 278 nm, λ_{min} = 240 nm; ^{1}H NMR ([D_6]DMSO): δ = 8.30 (t, 1H, *J* = 6.1 Hz, NH), 8.19 ("d", 2H, *J* = 8.8 Hz, Ph), 8.12 (s, 1H, H2), 7.58 (d, 2H, *J* = 8.8 Hz, Ph), 7.43 (d, 1H, *J* = 3.7 Hz, H6), 6.69 (d, 1H, *J* = 3.5 Hz, H5), 6.04 (d, 1H, *J* = 6.3 Hz, H1′), 5.28–5.30 (m, 2H, 2×OH), 5.14 (s, 1H, OH), 4.80–4.91 (m, 2H, CH_2), 4.45 ("d", 1H, *J* = 4.4 Hz, H2′), 4.11–4.13 (m, 1H, H3′), 3.92 (q, 1H, *J* = 3.5 Hz, H4′), 3.64 (dt, 1H, *J* = 3.9, 11.8 Hz, H5′), 3.52–3.57 ppm (m, 1H, H5″); ^{13}C NMR ([D_6]DMSO/D_2O): δ = 155.8, 151.0, 148.8, 147.9, 146.3, 127.9 (Ph), 123.4 (Ph), 122.8 (C6), 103.6, 99.3 (C5), 87.5 (C1′), 84.9 (C4′), 73.5 (C2′), 70.4 (C3′), 61.6 (C5′), 42.7 ppm (CH_2); MS (ESI): *m/z* 402 [*M*+H]$^+$ (100%); Anal. calcd for $C_{18}H_{19}N_5O_6 \cdot 1.25\,H_2O$ (423.89): C, 51.00; H, 5.11; N, 16.52; found: C, 51.05; H, 5.06; N, 16.03.

Method B

Step a. Ac_2O (377 μL, 408 mg, 4 mmol) was added to a stirred suspension of tubercidin (**1a**; 266 mg, 1 mmol) in dry pyridine (5 mL) at 0 °C (ice bath), and the solution was stirred at 0 °C for 12 h and then at RT for 9 h (total reaction time: 21 h). MeOH was added, and the reaction mixture was stirred at RT for a further 30 min. The volatiles were removed in vacuo (<25 °C) with coevaporation using MeOH. The resulting gum was partitioned between $CHCl_3$ (50 mL) and 2% aq AcOH (50 mL), and the aqueous layer was extracted with $CHCl_3$ (2×10 mL). The combined organic phase was washed sequentially with saturated aq $NaHCO_3$ (50 mL) and brine (50 mL), dried ($MgSO_4$), filtered and concentrated in vacuo. Purification of the residue using column chromatography (100%, EtOAc) gave 2′,3′,5′-tri-*O*-acetyltubercidin (**3**) as a colorless foam (337 mg, 86%); characterization data were in agreement with those previously reported.[33]

Step b. $NaNO_2$ (66 mg, 0.95 mmol) was added to a solution of **3** (150 mg, 0.38 mmol) in freshly prepared ~55% HF in pyridine[31b] (1.9 mL) at −10 °C in a sealed polypropylene vessel. The mixture was stirred at −10 °C for 15 min, and TLC showed almost complete conversion to a less polar product. Ice/H_2O (50 mL) was added, and the mixture was extracted with CH_2Cl_2 (4×25 mL). The combined organic phase was washed sequentially with saturated aq $NaHCO_3$ (50 mL) and brine (50 mL), and dried (Na_2SO_4), filtered and concentrated in vacuo. Purification of the resulting brown oil using column chromatography (30%, EtOAc/hexane) gave 7-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-4-fluoropyrrolo[2,3-*d*]pyrimidine (**4**) as a colorless oil (124 mg, 82%): UV (MeOH): λ_{max} = 258 nm, λ_{min} = 233 nm; ^{1}H NMR ($CDCl_3$): δ = 8.53 (d, 1H, $^4J_{H\text{-}F}$ = 0.5 Hz, H2), 7.37 (d, 1H, *J* = 3.8 Hz, H8), 6.66 (d, 1H, *J* = 3.8 Hz, H7), 6.45 (d, 1H, *J* = 6.0 Hz, H1′), 5.74 (t, 1H, *J* = 5.8 Hz, H2′), 5.55 (dd or m, 1H, *J* = 4.1, 5.6 Hz, H3′), 4.32–4.42 (m, 3H, H4′, H5′, H5″), 2.12 (s, 3H, CH_3), 2.13 (s, 3H, CH_3), 2.02 ppm (s, 3H, CH_3); ^{19}F NMR ($CDCl_3$): δ = −64.81 ppm (s); ^{13}C NMR ($CDCl_3$): δ = 170.2, 169.6, 169.4 (3×C=O), 162.3 (d, $^1J_{C\text{-}F}$ = 253.8 Hz, C4), 155.1 (d, $^3J_{C\text{-}F}$ = 11.9 Hz, C7a), 151.1 (d,

$^3J_{C\text{-}F}$ = 14.5 Hz, C2), 125.7 (d, $^4J_{C\text{-}F}$ = 2.6 Hz, C6), 105.4 (d, $^2J_{C\text{-}F}$ = 33.4 Hz, C4a), 99.6 (d, $^3J_{C\text{-}F}$ = 4.9 Hz, C5), 86.1 (C1′), 79.9 (C4′), 73.1 (C2′), 70.1 (C3′), 63.3 (C5′), 20.7 (CH_3), 20.5 (CH_3), 20.3 ppm (CH_3); MS (ESI): *m/z* 396 $[M+H]^+$ (100%).

Step c. Freshly distilled Et_3N (113 μL, 82 mg, 0.81 mmol) was added to a stirred suspension of **4** (93 mg, 0.23 mmol) and 4-nitrobenzylamine hydrochloride (67 mg, 0.35 mmol) in MeOH (3 mL), and the mixture was stirred for 5 h at RT. The volatiles were evaporated in vacuo, and the residue was purified by column chromatography (50%, EtOAc/hexane) to give 2′,3′,5′-tri-*O*-acetyl-4-*N*-(4-nitrobenzyl)-tubercidin (**5**) as a colorless oil (58 mg, 47%): UV (MeOH): λ_{max} = 277 nm, λ_{min} = 238 nm; ^{1}H NMR ($CDCl_3$): δ = 8.36 (s, 1H, H2), 8.17 (d, 2H, *J* = 8.7 Hz, Ph), 7.51 (d, 2H, *J* = 8.7 Hz, Ph), 7.12 (d, 1H, *J* = 3.77 Hz, H6), 6.42–6.46 (dd, 2H, *J* = 3.8, 6.0 Hz, H1′, H5), 5.73 (t, 1H, *J* = 5.8 Hz, H2′), 5.54–5.59 (m, 2H, H3′, NH), 4.95 (d, 2H, *J* = 6.1 Hz, CH_2), 4.31–4.40 (m, 3H, H4′, H5′, H5″), 2.14 (s, 6H, 2×CH_3), 2.04 ppm (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ = 170.4, 169.7, 169.5 (3× C=O), 156.0 (C4), 152.3 (C2), 150.8 (C7a), 147.3 (Ph), 146.7 (Ph), 128.0 (Ph), 123.9 (Ph), 121.5 (C6), 103.8 (C4a), 99.4 (C5), 85.4 (C1′), 79.5 (C4′), 73.1 (C2′), 70.8 (C3′), 63.5 (C5′), 44.2 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 ppm (CH_3); MS (ESI): *m/z* 528 $[M+H]^+$ (100%).

Step d. A saturated (at 0 °C) solution of NH_3 in MeOH (5 mL) was added to a stirred solution of **5** (54 mg, 0.1 mmol) in MeOH (1 mL), and the mixture was stirred at RT for 20 h. The volatiles were evaporated in vacuo, and the residue was purified by column chromatography (2→4% gradient, EtOAc/*i*PrOH/H_2O (4:1:2) in EtOAc) to give **2b** as a yellow oil (35 mg, 85%): see method A for characterization data.

5-Carboxamido-4-(4-nitrobenzylamino)-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine (2c): Sangivamycin (**1b**; 155 mg, 0.5 mmol) was treated with 4-nitrobenzyl bromide (162 mg, 1.5 mmol) for 26 h at 40 °C as described for the conversion of **1a**→**2a**. The alkylated intermediate was then dissolved in MeOH (12 mL), treated with Me_2NH in THF (2 M, 6 mL), and stirred for 45 h at RT. The reaction was then treated with additional Me_2NH in THF (2 M, 2 mL), and the mixture was heated at reflux while stirring for 32 h (total reaction time: 77 h). TLC showed ~80% conversion to the less polar product (**2c**). Volatiles were evaporated in vacuo to give a yellow solid. Recrystallization from EtOH gave **2c** as off-white crystals (101 mg, 45%): mp: 154–158 °C (dec.); UV (MeOH): λ_{max} = 286 nm (ε = 23100), λ_{min} = 229 nm (ε = 8700); ^{1}H NMR ([D$_6$]DMSO): δ = 10.29 (t, 1H, *J* = 6.0 Hz, NH), 8.17–8.21 (m, 4H, Ph, H2, H6), 8.11 (s, 1H, $CONH_2$), 7.59 (br d, 2H, *J* = 8.8 Hz, Ph), 7.47 (s, 1H, $CONH_2$), 6.06 (d, 1H, *J* = 6.0 Hz, H1′), 5.43 (d, 1H, 2′-OH, *J* = 6.3 Hz), 5.20 (d, 1H, *J* = 5.0 Hz, 3′-OH), 5.09 (t, 1H, *J* = 5.8 Hz, 5′-OH), 4.90 ("d", 2H, *J* = 6.2 Hz, CH_2), 4.37 (q, 1H, *J* = 5.8 Hz, H2′), 4.10 ("q", 1H, *J* = 4.5 Hz, H3′), 3.93 (q, 1H, *J* = 4.0 Hz, H4′), 3.61–3.67 (m, 1H, H5′), 3.53–3.58 ppm (m, 1H, H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 166.5, 156.5, 152.6, 150.4, 148.0, 146.4, 128.0 (Ph), 125.7, 123.6, 110.9, 101.7, 87.2 (C1′), 85.3 (C4′), 73.9 (C2′), 70.5 (C3′), 61.9 (C5′), 42.9 ppm (CH_2); MS (ESI): *m/z* 445 $[M+H]^+$ (100%); Anal. calcd for $C_{19}H_{20}N_6O_7$·1.5 H_2O (471.42): C, 48.41; H, 4.92; N, 17.83; found: C, 48.59; H, 4.66; N, 17.65.

5-Cyano-4-(4-nitrobenzylamino)-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (2d): Toyocamycin (**1c**; 146 mg, 0.5 mmol) was heated with 4-nitrobenzyl bromide (364 mg, 1.5 mmol) in dried DMF (3 mL) at 40 °C for 63 h (TLC showed ~85% conversion to a less polar product). The volatiles were evaporated under vacuum (< 40 °C) to ~1 mL, and this material was added dropwise to vigorously stirred dry acetone (20 mL). Et_2O (40 mL) was added to the stirred suspension, which was then chilled at 0 °C for 20 min. The precipitate was collected by vacuum filtration, and recrystallization from MeOH gave **2d** as colorless crystals (99 mg, 46%): mp: 214–216 °C; UV (MeOH): λ_{max} = 274, 236 nm (ε = 22100, 18900), λ_{min} = 250, 228 nm (ε = 12800, 18000); ^{1}H NMR ([D$_6$]DMSO): δ = 8.35 (s, 1H, H2), 8.19–8.23 (m, 3H, H6, Ph), 7.59 (d, 2H, *J* = 8.6 Hz, Ph), 6.87 (s, 1H, NH), 5.93 (d, 1H, *J* = 5.6 Hz, H1′), 5.47 (d, 1H, *J* = 6.0 Hz, 2′-OH), 5.35 ("s", 2H, CH_2), 5.21 (d, 1H, *J* = 5.0 Hz, 3′-OH), 5.09 (t, 1H, *J* = 5.4 Hz, 5′-OH), 4.31 (q, 1H, *J* = 5.5 Hz, H2′), 4.08 (q, 1H, *J* = 4.6 Hz, H3′), 3.92 (q, 1H, *J* = 3.7 Hz, H4′), 3.62–3.68 (m, 1H, H5′), 3.53–3.58 ppm (m, 1H, H5″); ^{13}C NMR ([D$_6$]DMSO/D_2O): δ = 152.6, 149.4, 146.7, 144.9, 142.7, 129.4, 128.5 (Ph), 123.5 (Ph), 115.2, 105.0, 87.7 (C1′), 85.8 (C4′), 85.5, 74.6 (C2′), 70.1 (C3′), 61.1 (C5′), 48.8 ppm (CH_2); MS (ESI): *m/z* 427 $[M+H]^+$ (100%); Anal. calcd for $C_{19}H_{18}N_6O_6$·0.5 H_2O (435.39): C, 52.41; H, 4.40; N, 19.30; found: C, 52.17; H, 4.29; N, 19.30.

6-*N*-[*N*-phenylcarbamoyl]adenosine (9a)

Step a. A solution of phenyl isocyanate (35 μL, 39 mg, 0.32 mmol) and 2′,3′,5′-tri-*O*-acetyladenosine (**7**; 106 mg, 0.27 mmol) in CH_2Cl_2 (5 mL) was stirred at RT under N_2 for 48 h. An additional portion of phenyl isocyanate (35 μL, 39 mg, 0.324 mmol) was then added, and stirring was continued for 20 h (total reaction time: 68 h). The reaction mixture was partitioned between saturated aq $NaHCO_3$ (25 mL) and $CHCl_3$ (25 mL), and the organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification by column chromatography (70%, EtOAc/hexane) gave 2′,3′,5′-tri-*O*-acetyl-6-*N*-[*N*-phenylcarbamoyl]adenosine (**8a**) as a pale-yellow solid (111 mg, 80%): ^{1}H NMR ($CDCl_3$): δ = 11.89 (s, 1H, NH), 9.40 (s, 1H, NH), 8.62 (s, 1H, H2), 8.56 (s, 1H, H8), 7.63 ("d", 2H, *J* = 7.6 Hz, Ph), 7.35 ("t", 2H, *J* = 7.9 Hz, Ph), 7.11 ("t", 1H, *J* = 7.4 Hz, Ph), 6.25 (d, 1H, *J* = 5.4 Hz, H1′), 6.03 (t, 1H, *J* = 5.5 Hz, H2′), 5.69 ("dd", 1H, *J* = 4.4, 5.4 Hz, H3′), 4.37–4.48 (m, 3H, H4′, H5′, H5″), 2.15 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 2.07 (s, 3H, CH_3).

Step b. A saturated (at 0 °C) solution of NH_3 in MeOH (6 mL) was added to a solution of **8a** (103 mg, 0.2 mmol) in MeOH (3 mL), and the mixture was stirred at RT for 2 h. The volatiles were evaporated, and the residual solid was recrystallized from MeOH to give **9a** as a white powder (66 mg, 85%): mp: 193–195 °C; UV (MeOH): λ_{max} = 279 nm (ε = 28250), λ_{min} = 241 nm (ε = 9200); ^{1}H NMR ([D$_6$]DMSO): δ = 11.76 (s, 1H, NH), 10.17 (s, 1H, NH), 8.72 (s, 1H, H2), 8.69 (s, 1H, H8), 7.63 (d, 2H, *J* = 7.7 Hz, Ph), 7.36 (t, 2H, *J* = 7.9 Hz, Ph), 7.10 (t, 1H, *J* = 7.4 Hz, Ph), 6.01 (d, 1H, *J* = 5.6 Hz, H1′), 5.54 (d, 1H, *J* = 6.0 Hz, 2′-OH), 5.24 (d, 1H, *J* = 5.0 Hz, 3′-OH), 5.14 (t, 1H, *J* = 5.5 Hz, 5′-OH), 4.61 (q, 1H, *J* = 5.6 Hz, H2′), 4.19 (q, 1H, *J* = 4.7 Hz, H3′), 3.99 (q, 1H, *J* = 3.8 Hz, H4′), 3.67–3.73 (m, 1H, H5′), 3.55–3.61 ppm (m, 1H, H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 150.83 (C= O), 150.81 (C2), 150.6, 150.0, 142.4 (C8), 138.4, 128.9 (Ph), 123.2 (Ph), 120.6, 119.4 (Ph), 87.7 (C1′), 85.7 (C4′), 73.8 (C2′), 70.3 (C3′), 61.3 ppm (C5′); MS (ESI): *m/z* 387 $[M+H]^+$ (100%); Anal. calcd for $C_{17}H_{18}N_6O_5$·0.5 H_2O (395.37): C, 51.64; H, 4.84; N, 21.26; found: C, 51.38; H, 4.77; N, 21.02.

6-*N*-[*N*-(4-Methoxyphenyl)carbamoyl]adenosine (9b)

Step a. A solution of **7** (197 mg, 0.5 mmol) and 4-methoxyphenyl isocyanate (78 μL, 90 mg, 0.6 mmol) in CH_2Cl_2 (5 mL) was stirred at RT under N_2 for 12 h. Additional 4-methoxyphenyl isocyanate (78 μL, 90 mg, 0.6 mmol) was then added, and stirring was continued for 12 h (total reaction time: 24 h). The reaction mixture was partitioned between saturated aq $NaHCO_3$ (25 mL) and $CHCl_3$ (25 mL), and the organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give 2′,3′,5′-tri-*O*-acetyl-6-*N*-[*N*-(4-methoxyphenyl)carbamoyl]adenosine (**8b**) as a pale-yellow solid of sufficient purity for NMR characterization and use in the next step

(260 mg, 96%): ^{1}H NMR ($CDCl_3$): δ = 11.45 (s, 1H, NH), 8.62 (s, 1H, H2), 8.59 (s, 1H, H8), 8.12 (s, 1H, NH), 7.53 ("d", 2H, J = 8.9 Hz, Ph), 6.91 ("d", 2H, J = 9.0 Hz, Ph), 6.21 (d, 1H, J = 5.3 Hz, H1′), 5.97 (t, 1H, J = 5.4 Hz, H2′), 5.66 ("t", 1H, J = 4.8 Hz, H3′), 4.38–4.48 (m, 3H, H4′, H5′, H5″), 2.16 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.10 ppm (s, 3H, CH_3).

Step b. A saturated (at 0 °C) solution of NH_3 in MeOH (6 mL) was added to a solution of crude **8b** (260 mg, 0.5 mmol) in MeOH (3 mL), and the mixture was stirred at RT for 2 h. The volatiles were evaporated, and the residue was recrystallized from MeOH to give **9b** as a white powder (170 mg, 80%): mp: 181–182 °C; UV (MeOH): λ_{max} = 280 nm (ε = 21300), λ_{min} = 247 nm (ε = 11100); ^{1}H NMR ([D$_6$]DMSO): δ = 11.59 (s, 1H, NH), 10.07 (s, 1H, NH), 8.70 (s, 1H, H2), 8.67 (s, 1H, H8), 7.53 ("d", 2H, J = 4.5 Hz, Ph), 6.94 ("d", 2H, J = 4.5 Hz, Ph), 6.01 (d, 1H, J = 5.7 Hz, H1′), 5.54 (d, 1H, J = 6.0 Hz, 2′-OH), 5.24 (d, 1H, J = 5.0 Hz, 3′-OH), 5.14 (t, 1H, J = 5.6 Hz, 5′-OH), 4.61 (q, 1H, J = 5.5 Hz, H2′), 4.18 (q, 1H, J = 4.8 Hz, H3′), 3.98 (q, 1H, J = 3.8 Hz, H4′), 3.75 (s, 3H, OCH_3), 3.67–3.72 (m, 1H, H5′), 3.55–3.61 ppm (m, 1H, H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 155.4 (C=O), 150.9 (C2), 150.8, 150.4, 149.8, 142.3 (C8), 131.1, 121.2 (Ph), 120.3, 114.1 (Ph), 87.7 (C1′), 85.6 (C4′), 73.7 (C2′), 70.1 (C3′), 61.1 (C5′), 55.2 ppm (OCH_3); MS (ESI): m/z 417 $[M+H]^+$ (100%); Anal. calcd for $C_{18}H_{20}N_6O_6 \cdot 1.5\,H_2O$ (443.41): C, 48.76; H, 5.23; N, 18.95; found: C, 48.54; H, 4.97; N, 18.85.

6-*N*-[*N*-(4-Nitrophenyl)carbamoyl]adenosine (9c)

Step a. A solution of 4-nitrophenyl isocyanate (54 mg, 0.33 mmol) and **7** (117 mg, 0.30 mmol) in CH_2Cl_2 (20 mL) was stirred at RT under N_2 for 17 h (TLC showed ~85% conversion to a less polar product), and then heated at reflux for 24 h (total reaction time: 41 h). The reaction mixture was partitioned between saturated aq $NaHCO_3$ (25 mL) and $CHCl_3$ (25 mL), and the organic layer was dried (Na_2SO_4), filtered and concentrated to give 2′,3′,5′-tri-*O*-acetyl-6-*N*-[*N*-(4-nitrophenyl)carbamoyl]adenosine (**8c**) as a pale-yellow solid of sufficient purity for NMR characterization and use in the next step (160 mg, 96%): ^{1}H NMR ([D$_6$]DMSO): δ = 12.13 (s, 1H, NH), 10.62 (s, 1H, NH), 8.75 (s, 1H, H2), 8.71 (s, 1H, H8), 8.27 (d, 2H, J = 9.2 Hz, Ph), 7.90 (d, 2H, J = 9.2 Hz, Ph), 6.33 (d, 1H, J = 5.3 Hz, H1′), 6.05 (d, 1H, J = 5.7 Hz, H2′), 5.65 (t, 1H, J = 5.4 Hz, H3′), 4.42–4.47 (m, 1H, H4′, H5′), 4.25–4.3 (m, 1H, H5″), 2.13 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 2.03 ppm (s, 3H, CH_3).

Step b. A saturated (at 0 °C) solution of NH_3 in MeOH (6 mL) was added to a solution of crude **8c** (160 mg, 0.29 mmol) in MeOH (3 mL), and the mixture was stirred at RT for 2 h. The volatiles were evaporated, and the residual solid was recrystallized from MeOH to give **9c** as an off-white powder (80 mg, 65%): mp: 202–208 °C; UV (MeOH): λ_{max} = 317, 278 nm (ε = 16200, 11300), λ_{min} = 286, 241 nm (ε = 10300, 5600); ^{1}H NMR ([D$_6$]DMSO): δ = 12.27 (s, 1H, NH), 10.61 (s, 1H, NH), 8.73 (s, 1H, H2), 8.72 (s, 1H, H8), 8.27 ("d", 2H, J = 9.2 Hz, Ph), 8.00 ("d", 2H, J = 9.2 Hz, Ph), 6.01 (d, 1H, J = 5.7 Hz, H1′), 5.52 ("s", 1H, 2′-OH), 5.24 (d, 1H, J = 14.1 Hz, 3′-OH), 5.14 ("s", 1H, 5′-OH), 4.61 ("s", 1H, H2′), 4.18 ("s", 1H, H3′), 3.98 (q, 1H, J = 3.8 Hz, H4′), 3.68–3.71 (m, 1H, H5′), 3.55–3.61 ppm (m, 1H, H5″); ^{1}H NMR ([D$_6$]DMSO/D_2O): δ = 8.71 (s, 1H, H2), 8.68 (s, 1H, H8), 8.24 ("d", 2H, J = 9.2 Hz, Ph), 7.88 ("d", 2H, J = 9.2 Hz, Ph), 6.00 (d, 1H, J = 5.7 Hz, H1′), 4.58 (t, 1H, J = 5.4 Hz, H2′), 4.15 ("t", 1H, J = 4.3 Hz, H3′), 3.99 (q, 1H, J = 3.7 Hz, H4′), 3.68 (dd, 1H, J = 3.8, 12.1 Hz, H5′), 3.57 ppm (dd, 1H, J = 3.9, 12.2 Hz, H5″); ^{13}C NMR ([D$_6$]DMSO): δ = 150.9, 150.72, 150.66, 149.4, 144.5, 142.6 (C8), 142.2, 125.0, 120.5, 119.1, 87.7 (C1′), 85.6 (C4′), 73.7 (C2′), 70.1 (C3′), 61.1 ppm (C5′); MS (ESI): m/z 432 $[M+H]^+$ (100%); Anal. calcd for $C_{17}H_{17}N_7O_7 \cdot H_2O$ (449.37): C, 45.44; H, 4.26; N, 21.82; found: C, 45.10; H, 3.96; N, 21.59.

Cytostatic activity assays

All assays were performed in 96-well microtiter plates. Each well containing 5–7.5×10^4 tumor cells (200 μL) was treated with a given amount of test compound in DMSO in cell culture medium at fivefold dilutions starting at 250 μM as the highest compound concentration. Cells were allowed to proliferate for 48 h (murine leukemia; L1210), 72 h (human T-lymphocytes; CEM), 96 h (human cervix carcinoma; HeLa), 144 h (human prostate adenocarcinoma; PC-3), or 168 h (human kidney; Caki-1) at 37 °C in a humidified 5% CO_2 controlled atmosphere. At the end of the incubation period, cells were counted in a Coulter counter. The IC_{50} value was defined as the concentration of the compound that inhibited cell proliferation by 50%. Data are the mean ± SD of at least 2–3 independent experiments.

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