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Synthesis and Antihistaminic Activity of 2-Guanadino-3-cyanopyridines and Pyrido[2,3-*d*]-pyrimidines

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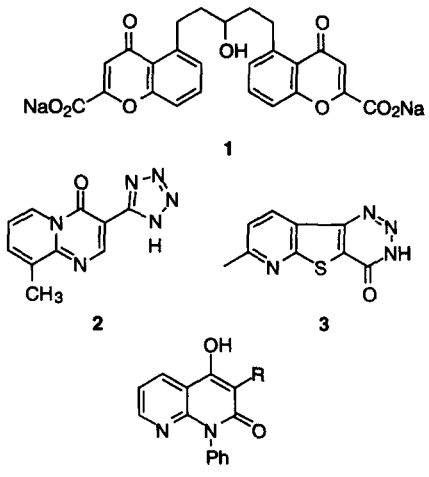
Abstract—2-Guanadino-3-cyanopyridines **8–33** and pyrido[2,3-*d*]-pyrimidines **35–52** were synthesized by nucleophilic displacement and cyclization of the chloroamidines **6a–d** easily obtained by reaction of 2-aminocyanopyridines **5a–d** with phosgene iminium chloride and their action on the release of histamine by mast cells examined under immunological and chemical stimulus, with and without pre-incubation. Several 2-guanadino-3-cyanopyridines and pyrido[2,3-*d*]-pyrimidines are shown to be inhibitors of the release of histamine when stimulated with ovoalbumin as antigen or with polymer 48/80 as chemical stimulus. Guanadino-3-cyanopyridine **30** and pyrido[2,3-*d*]-pyrimidine **49** are the more active of all, inhibiting the release of histamine in all the conditions tested (30–60% inhibition). Guanadinocyanopyridines **15**, **17**, and **19** are very potent stimulators of the release of histamine (150–300%) while pyrido[2,3-*d*]-pyrimidines are mostly inactive. Compounds **28** and **14** present moderate in vitro cytotoxic activity against P-388, A-549, HT-29, and MEL-28 cell lines. © 1997 Elsevier Science Ltd.

Introduction

The release of vasoactive mediators including histamine, leukotrienes and prostaglandins have been implicated in a variety of clinical manifestations including allergic bronchial asthma, rhinitis, allergic conjunctivitis, atopic eczema and allergic reactions to foods.[1] Bronchial asthma is a chronic debilitating disease, which in its severe forms can be life-threatening. At present, four classes of drugs have been employed to combat the symptoms of this disease: β-sympathomimetic agents, bronchodilators, anti-allergic agents and corticosteroids. One of the most useful drugs for the treatment of asthma is disodium cromoglycate (DSCG, **1**),[2] whose clinical effectiveness has been attributed to its ability to inhibit, both in vivo and in vitro, IgE-mediated secretion of vasoactive mediators from mast cells.[3] However, one main disadvantage of this drug as a prophylactic therapeutic agent is its ineffectiveness following oral administration and it must thus be used as an insufflated powder.

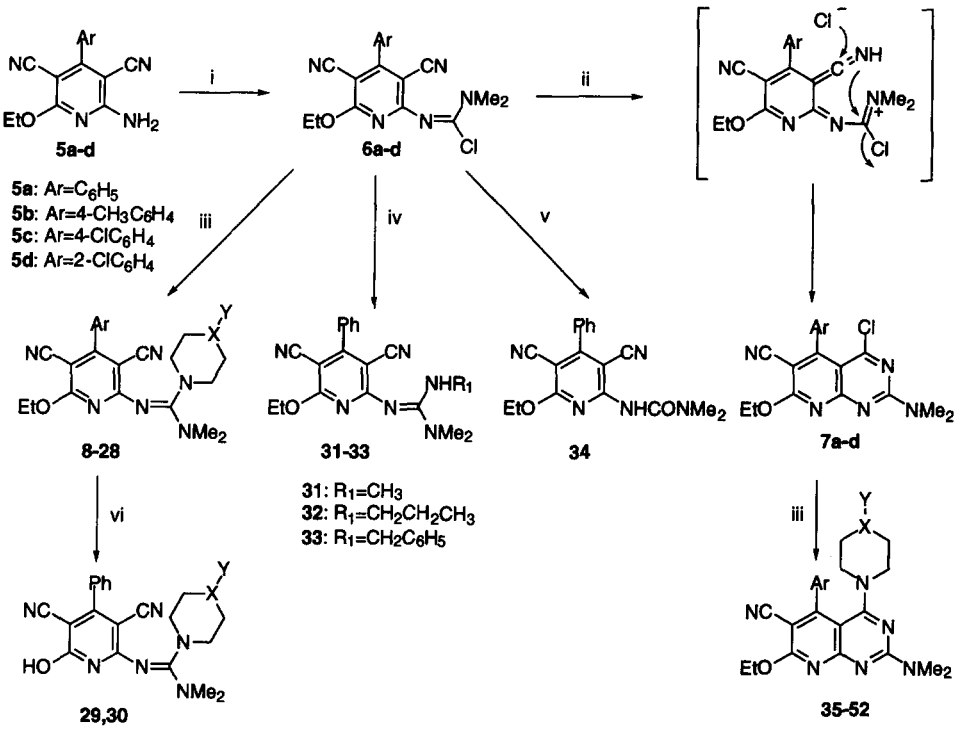
Since the discovery of DSCG (**1**) there has been a continuous and widespread interest in the synthesis of orally active DSCG-like effective inhibitors, and several new antiallergic agents have been discovered.[4] Most of the orally active compounds reported are carboxylic acid derivatives or tetrazoles,[5] such as 9-methyl-3-(1*H*-tetrazol-5-yl)-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (**2**) that is used clinically as its potassium salt (pemirolast potassium) for the oral treatment of asthma.[6] A few orally active compounds with other heterocyclic systems are the pyridothienotriazine (**3**) (RHC2963), which inhibit the immunologically-induced release of histamine[7] and naphtyridines **4a** (Sch 33303) and **4b** (Sch 37224) reported by Sherlock et al.[8] and Kreutner et al.,[9] respectively, which show potent inhibition of the release of leukotrienes.

In this paper we describe the synthesis and antihistaminic activity of a series of compounds derived from two related heterocyclic systems: the guanadinopyridines and the pyridopyrimidines. For this study we examined pyridopyrimidines **35–52** and their open



1 2 3

Chart 1.



Scheme 1. (i) $Cl_2CN^+Me_2Cl^-$, 1,2-dichloroethane, reflux, 20 h; (ii) $HCl_{(G)}$, 1,2-dichloroethane, rt; (iii) secondary amine, ethanol, reflux; (iv) primary amine, ethanol, reflux; (v) NH_4OH, acetonitrile, rt; (vi) NaOH, ethanol, reflux.

precursors the 2-guanadino-3-cyanopyridines **8–33** (Scheme 1 and Tables 1 and 2), where structural elements X, Y and Ar, were systematically altered.

The antihistaminic activity was tested on rat mast cells because this cellular model has been reported to have pharmacological behaviour similar to human skin mast cells.[10] The experiments were carried out measuring both the inhibition and the stimulation of histamine release with immunological and non-immunological stimulus, (ovoalbumin and the commercially available polymer 48/80, respectively). Since the action of DSCG depends on whether the drug is added simultaneously or previously to the stimulus, the response of every compound was measured with simultaneous addition with the stimulus and after pre-incubation of the cells with the drug before addition of the stimulus.

In order to determine if cytoxicity may be a problem in the use of these compounds as antihistaminics, their in vitro cytotoxicity against murine P388 (lymphoid neoplasm) and human A-549 (colon carcinoma), HT-29 (lung carcinoma), and MEL-28 (skin carcinoma) cell lines was determined.

Chemistry

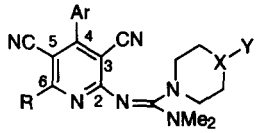
The title compounds were prepared via synthetic intermediates **6** (Scheme 1), which contain the adequate functionality to undergo substitution by nucleophiles, giving **8–33** or to produce pyridopyrimidines **35–52** by intramolecular cyclization.

The starting 2-amino-3-cyanopyridines **5a–d** were readily obtained by a previously described procedure.[11] In our scheme, the conversion of **5** into **6** involves the introduction of one additional carbon that must conserve its electrophile character in **6**. To this end, we selected phosgene iminium chloride, a well-known multiple electrophilic one carbon atom synthon,[12-14] as the reagent of choice. In this way, 2-amino-3-cyanopyridines **5a–d**, were made to react with *N,N*-dimethyldichloromethylene iminium chloride in refluxing 1,2-dichloroethane affording the 2-chloroamidines **6a–d**. Treatment of **6** with primary or secondary amines produced the expected halide displacement and 2-guanadino-3-cyanopyridines **8–33** were obtained in good yields (54–85%). When compounds **6a–d** were treated with dry hydrogen chloride, they underwent intramolecular attack by the cyano group and cyclization to the corresponding fused heterocyclic compounds **7a–d**.

It is known that dichloromethylene iminium salts do not normally react with the nitrile group so the formation of pyrido[2,3-*d*]-pyrimidines **7** from **6** can be assumed to proceed through the corresponding chloroiminium chloride.[15]

Aromatic nucleophilic displacement of the chlorine atom in **7** was achieved by reaction with nucleophiles and resulted in the formation of substituted products **35–52** in good yields (65–96%). One-pot high yield (80–90%) synthesis of pyridopyrimidines **7** from **5** was carried out by refluxing **5** and phosgene iminium

Table 1. Physicochemical data for 2-guanadino-3-cyanopyridines **8–30**



Compound[a]	Ar	R	X	Y	Mp (°C)	Yield (%)	Formula[b]
8	C_6H_5	EtO	CH	CH_3	173–175	68[c]	$C_{24}H_{28}N_7O$
9	C_6H_5	EtO	N	CH_3	163–164	76[d]	$C_{23}H_{27}N_7O$
10	C_6H_5	EtO	N	$CH_2C_6H_5$	177–179	78[d]	$C_{29}H_{31}N_7O$
11	C_6H_5	EtO	N	Piperonyl	203–205	78[d]	$C_{30}H_{31}N_7O_3$
12	4-$CH_3C_6H_4$	EtO	CH	CH_3	187–189	71[e]	$C_{25}H_{30}N_6O$
13	4-$CH_3C_6H_4$	EtO	N	CH_3	194–195	84[d]	$C_{24}H_{29}N_7O$
14	4-$CH_3C_6H_4$	EtO	N	4-$CH_3COC_6H_4$	234–236	54[f]	$C_{31}H_{33}N_7O_2$
15	4-$CH_3C_6H_4$	EtO	N	$CH_2C_6H_5$	114–116	69[f]	$C_{30}H_{33}N_7O$
16	4-$CH_3C_6H_4$	EtO	N	Piperonyl	117–119	73[g]	$C_{31}H_{33}N_7O_3$
17	4-ClC_6H_4	EtO	N	CH_3	176–178	85[d]	$C_{23}H_{26}N_7OCl$
18	4-ClC_6H_4	EtO	N	4-$CH_3COC_6H_4$	268–270	62[f]	$C_{30}H_{30}N_7O_2Cl$
19	2-ClC_6H_4	EtO	N	CH_3	165–167	78[d]	$C_{23}H_{26}N_7OCl$
20	2-ClC_6H_4	EtO	N	4-$CH_3COC_6H_4$	198–200	59[d]	$C_{30}H_{30}N_7O_2Cl$
21	C_6H_5	EtO	O	—	191–193	80[h]	$C_{22}H_{24}N_6O_2$
22	C_6H_5	EtO	N	C_6H_5	215–217	73[d]	$C_{28}H_{29}N_7O$
23	C_6H_5	EtO	N	3-$CF_3C_6H_4$	189–191	68[i]	$C_{29}H_{28}N_7OF_3$
24	C_6H_5	EtO	N	CO_2Et	229–231	77[d]	$C_{25}H_{29}N_7O_3$
25	C_6H_5	EtO	N	$CH_2CON(CH_2)_4$	105–107	67[j]	$C_{29}H_{34}N_6O_2$
26	C_6H_5	EtO	N	$COCH_3$	186–188	71[d]	$C_{24}H_{27}N_7O_2$
27	C_6H_5	EtO	CH	C_6H_5	186–188	76[d]	$C_{29}H_{30}N_6O$
28	C_6H_5	EtO	N	4-$CH_3COC_6H_4$	221–223	64[i]	$C_{30}H_{31}N_7O_2$
29	C_6H_5	OH	N	$CH_2C_6H_5$	239–241	71[d]	$C_{27}H_{27}N_7O$
30	C_6H_5	OH	N	4-$CH_3COC_6H_4$	255–257	80[f]	$C_{28}H_{27}N_7O_2$

[a]All spectra data were consistent with the assigned structures.
[b]All compounds analysed for C, H, N; analytical results were within ± 0.4% of theoretical values.
[c]Purified by medium-pressure column chromatography using dichloromethane:hexane (1:1, v/v) as eluent.
[d]Recrystallized from ethanol.
[e]Purified by medium-pressure column chromatography using dichloromethane:hexane (1:2, v/v) as eluent.
[f]Recrystallized from ethanol:dichloromethane.
[g]Purified by medium-pressure column chromatography using dichloromethane:ethanol (99:1, v/v) as eluent.
[h]Recrystallized from ethanol:acetone.
[i]Purified by medium-pressure column chromatography using dichloromethane:hexane (3:1, v/v) as eluent.
[j]Purified by medium-pressure column chromatography using dichloromethane as eluent.

chloride in 1,2-dichloroethane for 1 h followed by treatment with hydrogen chloride.

All the compounds shown in Tables 1 and 2 gave satisfactory elemental analyses and spectral data (IR, MS, and ^1H and ^{13}C NMR) coherent with the structures proposed. Compounds **6a–d** show essentially the same UV as the starting **5**. In addition, signals due to the imino (n = 1620 cm^{-1} in the IR and at δ = 142.7–143.1 ppm in the ^{13}C NMR spectra) and cyano groups (two resonances at δ = 113.5–114.4 and δ = 114.7–115.3 ppm) were found. For their part, cyclic compounds **7a–d** and **35–52** show the UV typical of a pyridopyrimidine system, and one resonance for the cyano group (δ = 113.6–114.4 ppm) and one additional aromatic carbon in the ^{13}C NMR spectra.

Compounds **8–33** present in comparison with **6** similar UV spectra and ^{13}C NMR chemical shifts for the cyano group, but the imine carbon is shifted in **8–33** to >160 ppm in the ^{13}C NMR and to <1600 cm^{-1} in the IR, in coherence with the replacement of the chlorine in **6** for other substituents.

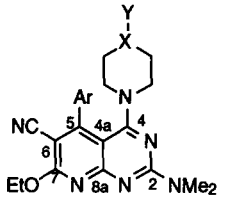
Biological results

The release of proinflamatory mediators can be induced by two different pathways: the antigenic pathway that requires the specific cross-linking of the FceRI receptors and (2) a non-IgE mediated pathway, which is related to G-protein-mediated receptors or G-proteins themselves, as is the case for polymer 48/80.[16] Since the effect of cromoglycate is different when added to the cells simultaneously or before the stimulus, depending on the type of stimulus, we evaluated guanadinopyridines **8–33** and pyridopyrimidines **35–52** as potential inhibitors or stimulants of histamine release in both conditions, that is by addition of the chemical before the stimulus (pre-incubation) or simultaneously. Selected results are shown in Figures 1–4. Compounds not included in those plots are inactive.

2-Guanadino-3-cyanopyridines, 8–34

The results of IgE-mediated stimulation of mast cells using ovoalbumin (Fig. 1) showed that several 2-guanadino-3-cyanopyridines (**10**, **13**, **16**, **18**, **27**, **28**, **29**

Table 2. Physicochemical data for pyrido[2,3-*d*]pyrimidines **35–52**



Compound[a]	Ar	X	Y	Mp (°C)	Yield (%)[a]	Formula[b]
35	C_6H_5	CH	CH_3	193–195	77[c]	$C_{24}H_{28}N_6O$
36	C_6H_5	N	CH_3	237–239	72[d]	$C_{23}H_{27}N_7O$
37	C_6H_5	N	4-$CH_3COC_6H_4$	225–227	65[c]	$C_{30}H_{31}N_7O_2$
38	C_6H_5	N	$CH_2C_6H_5$	255–257	80[e]	$C_{29}H_{31}N_7O$
39	C_6H_5	N	Piperonyl	247–249	73[e]	$C_{30}H_{31}N_7O_3$
40	C_6H_5	CH_2	—	216–218	88[c]	$C_{23}H_{26}N_6O$
41	C_6H_5	O	—	232–233	74[c]	$C_{22}H_{24}N_6O_2$
42	4-$CH_3C_6H_4$	CH	CH_3	164–166	81[c]	$C_{25}H_{30}N_6O$
43	4-$CH_3C_6H_4$	N	CH_3	177–179	77[c]	$C_{24}H_{29}N_7O$
44	4-$CH_3C_6H_4$	N	4-$CH_3COC_6H_4$	207–209	71[e]	$C_{31}H_{33}N_7O_2$
45	4-$CH_3C_6H_4$	N	$CH_2C_6H_5$	224–225	96[c]	$C_{30}H_{33}N_7O$
46	4-$CH_3C_6H_4$	N	Piperonyl	237–239	88[c]	$C_{31}H_{33}N_7O_3$
47	4-ClC_6H_4	CH	CH_3	216–217	75[c]	$C_{24}H_{27}N_6OCl$
48	4-ClC_6H_4	N	CH_3	245–247	70[c]	$C_{23}H_{26}N_7OCl$
49	4-ClC_6H_4	N	4-$CH_3COC_6H_4$	186–188	66[c]	$C_{30}H_{30}N_7O_2Cl$
50	2-ClC_6H_4	CH	CH_3	210–212	80[c]	$C_{24}H_{27}N_6OCl$
51	2-ClC_6H_4	N	CH_3	237–239	76[c]	$C_{23}H_{26}N_7OCl$
52	2-ClC_6H_4	N	4-$CH_3COC_6H_4$	175–176	70[d]	$C_{30}H_{30}N_7O_2Cl$

[a]All spectra data were consistent with the assigned structures.
[b]All compounds analysed for C, H, N; analytical results were within ± 0.4% of theoretical values.
[c]Recrystallized from ethanol.
[d]Purified by column chromatography with dichloromethane:ethanol (99:1, v/v) as eluent.
[e]Recrystallized from ethanol:dichlomethane.

and **30**) are inhibitors producing about 30% less histamine than the control (Fig. 1). Only one compound (**21**) shows greater inhibitory activity (50%) after pre-incubation. In most cases the inhibition was higher when the antigen is added simultaneously with the compound and lower after pre-incubation. A particular case is represented by compounds **28** and **29** whose responses changed dramatically depending on the mode of addition to the cells: They behaved as inhibitors when added simultaneously with the stimulus, but promoted the liberation of histamine when pre-incubated.

Analysis of the histamine released by chemically stimulated rat mast cells is shown in Figure 2. Several guanadino derivatives (**13**, **16**, **18**, **20**, **22**, **24**, **27**, **28** and **30**) are shown to produce inhibition greater than 30% when the polymeric compound 48/80 was added, either simultaneously or after pre-incubation with the drug. Compound **30** is the strongest inhibitor in this group (60% inhibition).

Comparison of the data in Figures 1 and 2 indicate that most of the 2-guanadino-3-cyanopyridines shown to be inhibitors in chemically stimulated experiments are also active under immunological stimulus (i.e. **13**, **16**, **18**, **20**, **22**, **27** and **30**). Nevertheless, in contrast to what has been observed in the IgE-mediated assays, the inhibition produced in the chemically stimulated cells is lower when the compound is added simultaneously and higher when it is pre-incubated.

Apart from that inhibitory action, three guanadino-cyanopyridines (**15**, **17** and **19**) were found to be very strong stimulants of histamine production under both chemical and immunological stimulus. Compound **17** is particularly active and produced an increase of histamine release of 170–300% depending on the particular conditions of the experiment. Structurally, the stimulants **15**, **17** and **19** are defined by Ar = 2 or 4-substituted phenyl ring, X = N and Y = CH_3 (**17** and **19**) or Y = benzyl (**15**). The absence of a substituent on the phenyl ring leads to the loss of activity (i.e. **10**, Ar = C_6H_5). As for the aromatic substituents, activity is linked to an *ortho*- or *para*-chloro as in **17** and **19**, but not to a *para*-Me (**13**). Comparison of **15** with **16** suggested that activity requires the absence of substitution in the aromatic part of the benzyl group.

Pyrido[2,3-*d*]pyrimidines 35–52

Among the pyrido[2,3-*d*]pyrimidines compounds **35**, **37**, **49**, **50**, **51** and **52** were shown to be moderate inhibitors (>25% inhibition) in pre-incubation immunologically-driven secretion assays (Fig. 3), but this activity is drastically reduced when the drug is added simultaneously with the antigen. In fact, compounds **35** and **37**

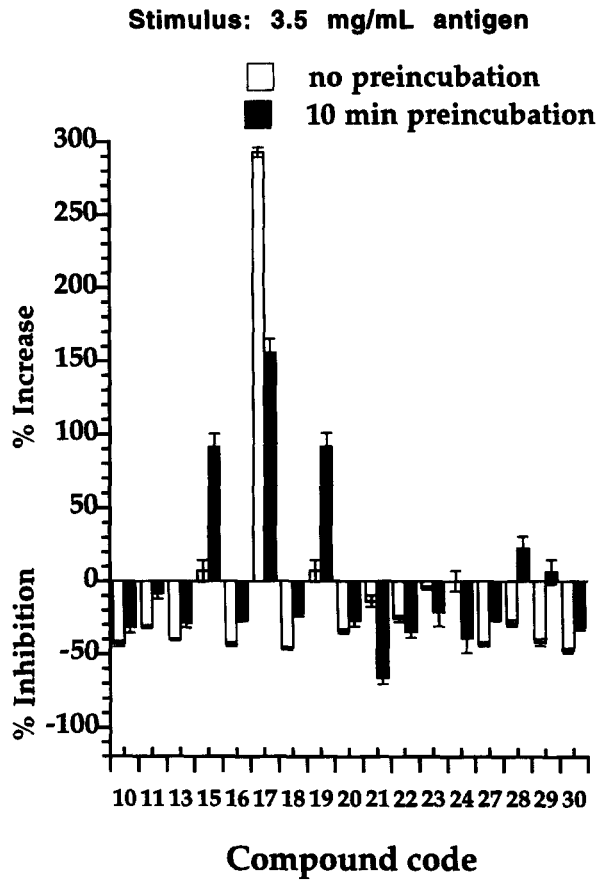


Figure 1. Histamine release in mast rat cells stimulated with 3.5 mg/mL of antigen (ovoalbumin) in the presence of 2-guanadino-3-cyanopyridines. The compounds were added to the cells simultaneously with the stimulus (no pre-incubation), or 10 min before the addition of the stimulus (10 min pre-incubation). The activity data are normalized to the response of the control experiments (20.1 ± 1.6% in the presence of 5 mg/mL of antigen).

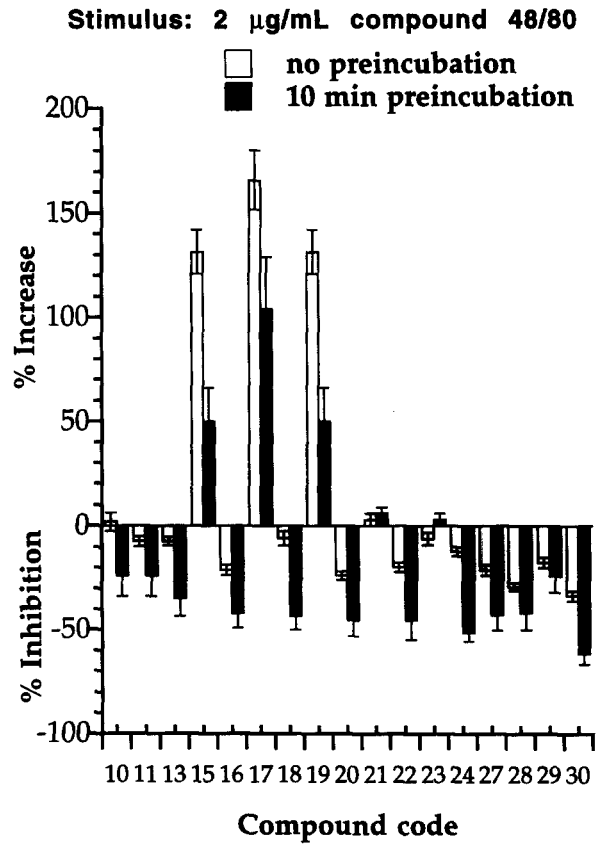


Figure 2. Histamine release in mast rat cells stimulated with 2 μg/mL of compound 48/80 in the presence of 2-guanadino-3-cyanopyridines. The compounds were added to the cells simultaneously with the stimulus (no pre-incubation), or 10 min before the addition of the stimulus (10 min pre-incubation). The activity data are normalized to the response of the control experiments (24.5 ± 1.5% in the presence of 2 μg/mL of compound 48/80).

are completely inactive and **52** behaves as an stimulant in this assay.

In the chemically stimulated experiments (Fig. 4) the stronger inhibitory action was found in **42**, **43** and **49**, which produced the release of only 30–50% of the histamine liberated by the control. Comparison of the data in Figures 3 and 4 indicates that only one of these pyrido[2,3-*d*]pyrimidines (**49**; X = N, Y = 4-$CH_3C_6H_4$) is an inhibitor under all the experimental conditions tested.

No potent histamine promoters were found among the pyridopyrimidines. Comparison with the data shown in Figures 1 and 2 show, in fact, the disappearance of the stimulant activity when the 2-guanadino-3-cyanopyridines are cyclized to pyrido[2,3-*d*]pyrimidines. Thus, compounds **45**, **48** and **51**, which share with the strong activants **15**, **17** and **19** the same substituents, are completely inactive. Only compounds **42** and **43** (inhibitors in chemical-stimulated experiments) produce a weak stimulation in the ovoalbumin-driven test. Similarly, compound **52**, a moderate inhibitor when pre-incubated with the antigen, acts as a weak stimulant (20%) in the simultaneous addition experiments.

Cytotoxic activity

The cytotoxicity of all the compounds prepared in this work was tested in vitro against standard cell lines and the IC_{50} of the more active ones are shown in Tables 3 and 4. The figures indicate that these compounds are not very potent and suggest that cytoxicity will not represent a problem in their use as antihistaminics.

Discussion

There is a good correlation between histamine released in vitro and in vivo by drugs when the mechanism to activate the cell is not immunological. Good examples of drugs that release histamine in vitro and in vivo, which may cause notable secondary effects, are morphine, tubocurarine, radiological contrast media,[17,18] etc. In the same way, although fewer examples are available, drugs such as β-blocking agents,[1] disodium cromoglycate,[20] nigellone,[21] azelastine,[22] tranilast[23] or

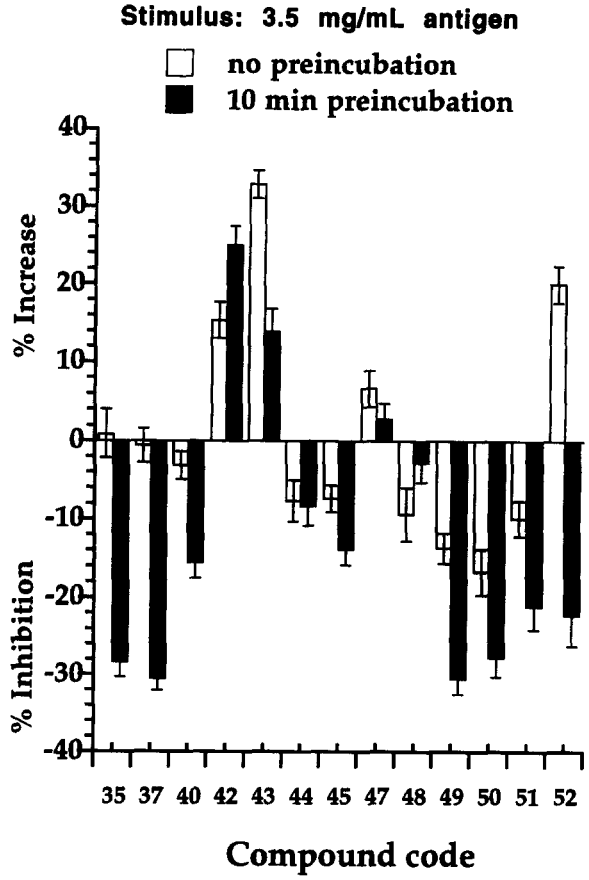


Figure 3. Histamine release in mast rat cells stimulated with 3.5mg/mL of antigen (ovoalbumin) in the presence of pyrido[2,3-*d*]pyrimidines. The compounds were added to the cells simultaneously with the stimulus (no pre-incubation), or 10 min before the addition of the stimulus (10 min pre-incubation). The activity data are normalized to the response of the control experiments (16.4 ± 1% in the presence of 5 mg/mL of antigen).

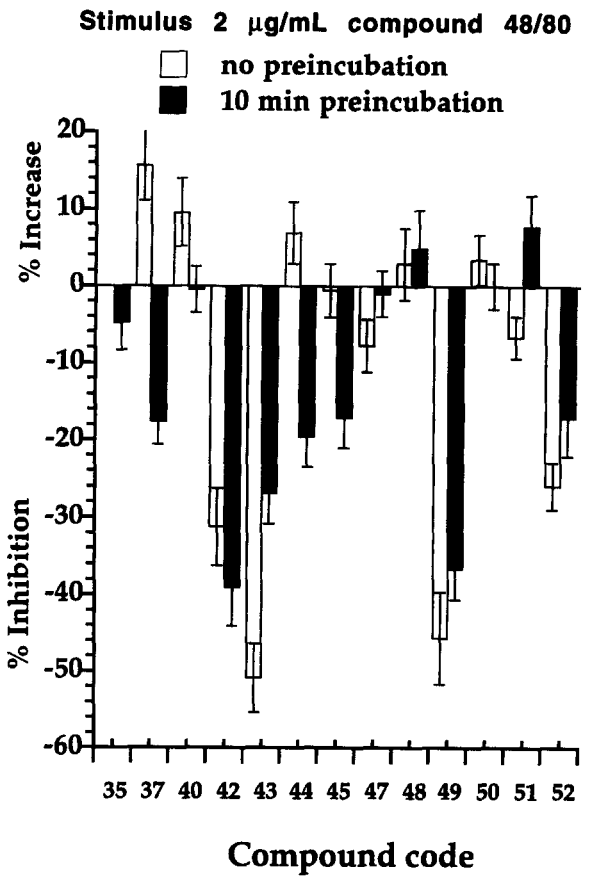


Figure 4. Histamine release in mast rat cells stimulated with 2 µg/mL of compound 48/80 in the presence of pyrido[2,3-*d*]pyrimidines. The compounds were added to the cells simultaneously with the stimulus (no pre-incubation), or 10 min before the addition of the stimulus (10 min pre-incubation). The activity data are normalized to the response of the control experiments (37.8 ± 2% in the presence of 2 µg/mL of compound 48/80).

nedocromil[24] inhibit histamine release in vitro and do the same in vivo.

Rat mast cells are a pharmacological model to human skin mast cells,[10] so our results constitute a good approach to the study of potentially interesting new inhibitors and provide clues to new chemical structures that may be used as anti-allergic drugs. The interest of this topic is obvious, because allergy, rhinitis or asthma are more prevalent in industrialized countries[25] and all the available treatments have several drawbacks. Sodium cromoglycate-type drugs should be the best since they inhibit the release of histamine, thus preventing the action of the large number of mediators from mast cells or basophils, but unfortunately sodium cromoglycate is not orally absorbed and several daily doses are needed.

The pre-incubation of sensitized mast cells with cromoglycate results in desensitization. We have tested each compound with and without pre-incubation to elucidate potential desensitization effects and establish a possible mechanistic analogy between cromoglycate and these compounds. On this basis the experiments suggest that several compounds (i.e. **10**, **13**, **16**, **18**, **20**, **27**, **30** and **48**) may act in a similar way to cromoglycate, although they present low desensitization levels due to pre-incubation. Nevertheless, we also observe that many actives produce the higher inhibition values after pre-incubation, suggesting a time-cumulative action that does not share the pattern of cromoglycate.

Several guanadinocyanopyridines (**16**, **18**, **20**, **22**, **27** and **30**) and one pyridopyrimidine (**49**) are good inhibitors in both immunological- and non-immunological-mediated stimulation, but some of the compounds tested are strong inhibitors only with immunological or with non-immunological activation, but not in both conditions (i.e. **42** and **43**). In any case, the potency of the active inhibitors is higher than that of sodium cromoglycate which has to be used in concentrations 100-fold higher (1000 µM) to inhibit histamine release in vitro[20] and this justifies the potential interest of these compounds.

Conclusions

In conclusion, the reaction of 2-amino-3-cyanopyridines with *N*,*N*-dimethyldichloromethylene iminium chloride

Table 3. Antitumoral activity of selected 2-guanadino-3-cyanopyridines

Compound	IC_{50} (μg/mL)			
	P-388	A-549	HT-29	MEL
8	2.5	2.5	2.5	2.5
10	1.2	1.2	2.5	2.5
11	2.5	1.2	2.5	5
12	2.5	2.5	2.5	5
13	>10	5	5	5
14	1	0.25	0.25	0.25
15	2.5	1.2	1.2	2.5
16	2.5	1.2	1.2	2.5
17	5	1	1	1
22	>10	>10	>10	>10
28	0.25	0.5	10	10
34	10	1	1	1

Table 4. Antitumoral activity of selected pyrido[2,3-*d*]pyrimidines

Compound	IC_{50} (μg/mL)			
	P-388	A-549	HT-29	MEL
36	1	2.5	2.5	2.5
37	5	1	5	2.5
39	10	>10	>10	>10
40	5	5	5	5
42	1	2.5	2.5	1
43	1	2.5	2.5	1
44	2.5	2.5	2.5	2.5
45	5	5	5	5
46	5	10	10	10
51	2.5	2.5	2.5	5

provides a good and general entry to 2-guanadino-3-cyanopyridines and to pyrido[2,3-*d*]pyrimidines. The antihistaminic activity of these two series of heterocycles has been evaluated in different conditions, and several compounds were found to be inhibitors under immunological and non-immunological conditions. Guanadinocyanopyridines (**16**, **18**, **20**, **22**, **27** and **30**) inhibit the release of histamine in all the conditions tested; compound **30** is the most potent in this series showing 30–60% inhibition. Among the pyridopyrimidines, only one compound (**49**) acts as inhibitor in all the conditions tested (30–40% inhibition). For their part, **15**, **17** and **19** are very strong stimulants. Compound **17** is particularly active; it induces the release of 150% and 300% of histamine in non-immunological and immunological experiments, respectively.

From a pharmacological point of view this immunological enhancement suggests that the mechanism of action is related to IgE receptor signalling. The interest of this in vitro antianaphylatic activity is stressed by the fact that other pyridopyrimidines have been shown to be effective in vivo too.[26,27]

A comparison of the activity of these compounds with that of cromoglycate **1** is illustrative of their potential interest. In fact, the inhibitory values of the more active ones are similar or higher than those reported for DSCG **1**, but our data have been obtained at much lower doses (100 μg/mL), which is one-fifth of the IC_{50} reported for DSCG (500 μg/mL), suggesting that these compounds are much more potent than DSCG. Although rat mast cells are considered to be good pharmacological cellular models of human skin cells, caution should be taken before any conclusion can be drawn for humans.[10]

The loss of the inhibitory effects observed when some active compounds are added before the stimulus can be explained as the result of a tachyphylatic process well documented for DSCG and potassium pemirolast,[28] but histamine release can be inhibited by drugs acting on different targets (calcium-channel blockers, β-agonists and enzyme inhibitors of phosphodiesterase, protein synthase and protein kinase)[29,30] and therefore further experiments are necessary to identify the signal transduction affected and the mechanism by which those compounds modify histamine release.

Some pyridopyrimidines have been reported in the literature for their antineoplasic effects.[31] Our results show that the pyridopyrimidines **35–52** are, in general, inactive but guanadinopyridines **28** and **14** (Tables 3 and 4) exhibit moderate in vitro antitumoral activity.

Further work is in progress with these and related heterocyclic systems to obtain information about the chemical structure that effectively modulates histamine release and to discover new chemicals useful as anti-allergic drugs.

Experimental

All reagents used in synthesis were commercial grade chemicals from freshly opened containers. The histamine releaser, compound 48/80, a condensation product of *N*-methyl-*p*-methoxy-phenethylamine, was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.); orthophtalaldehyde from Merck (Darmstadt, Germany), and *Bordetella pertussis* from Wako (Germany). Melting points were determined on a Büchi 510 apparatus and are reported uncorrected. Silica-gel 60 $HF_{254+366}$ for thin-layer chromatography and Silica gel 60 (230–400 mesh) for column chromatography were purchased from Merck. IR spectra were recorded as potassium bromide disks on a Perkin–Elmer 783 spectrophotometer. ^1H and ^{13}C NMR spectra were obtained in DMSO-d_6 on a Bruker AC 200F or a Bruker WM 250 instrument at room temperature. Mass spectra were obtained on a VG-QUATTRO or a Kratos MS-50 spectrometer. Fast atom bombardment mass spectra (FABMS) were determined using thioglycerol as matrix. Microanalyses for C, H and N were performed by the Elemental Analyses General Service of the University of La Coruña and were within 0.40 of the theoretical values.

Antihistaminic assays

Mast cell preparation. Mast cells were obtained by lavage of pleural and peritoneal cavities of Sprague–Dawley rats (200–400 g) as previously described.[32] Physiological saline composition was (mM): Na^+, 142.3; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 126.1; CO_3^{2-}, 22.85; $PO_4H_2^-$, 1.2; SO_4^{2-}, 1.2, giving a final osmotic pressure of 300 + 5 mOms/kg H_2O. Sucrose (1 mg/mL) and bovine serum albumin (BSA) were also added to the solution; the final pH being 7.0. The impurified cellular suspension contained 4–8% mast cells, with an average of $1.5–2 \times 10^6$ mast cells per rat.

Sensitization of rat mast cells. Sprague–Dawley rats weighting 200–300 g were sensitized by intramuscular injection in the back extremities of egg albumin (15 mg each rat) and adjuvant (9×10^9 killed *Bordetella pertussis* in each rat) in saline solution. Two weeks later, the rats were sacrificed and the mast cells isolated.

Cell incubation. A 25 μL quantity of a freshly prepared concentrated solution of each drug in dimethylsulfoxide was added to 0.9 mL of the incubation medium. When the medium reached 37 °C, 25 μL of cell suspension, containing $1–1.5 \times 10^5$ mast cells, were added and the cells were then incubated for 10 min (in the experiments performed without pre-incubation this step was omitted), and for 10 min further after addition of the stimulus. Since we used compound 48/80 as a reference drug each experiment was carried out with parallel controls of compound 48/80.

Incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 1000*g* for 5 min, the supernatants were collected and decanted into other tubes for histamine determination. We used trichloroacetic acid (final concentration of 7%) to precipitate the protein and avoid its interference with histamine determination (the presence of 1 mg/mL BSA interferes slightly with histamine determination). Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment. Spontaneous histamine release was never higher than 8%.

Histamine release assay. Histamine was assayed spectrofluorometrically both in the pellet (residual histamine) and supernatants (released histamine) by Shore's method[33] in a Perkin–Elmer LS-50 spectrofluorometer. Briefly, histamine reacts with 0.1% orthophtalaldehyde at alkaline pH, and the reaction is then stabilized by acidification, the fluorophore so obtained is stable for at least 2 h at room temperature. To ensure total histamine pellets were sonnicated for 60 s in 0.8 mL of 0.1 N HCl.

Results are expressed as a percentage of histamine released with respect to the total histamine content. Calculations were done substracting spontaneous histamine values from numerator and denominator. The trypan blue exclusion test was used in order to ensure that histamine release was not due to cytotoxicity.

Statistical analysis

Results were analysed using the Student's *t*-test for unpaired data. A probability level of 0.05 or less was used for statistical significance. Results are expressed as the mean ± SEM, and all experiments were at least repeated three times in duplicate.

We also carried out control experiments with cromoglycate. A maximum 40% inhibitory effect was obtained with cromoglycate at 500 μg/mL, while at 100 μg/mL (the concentration used for all the compounds in this study) there was no inhibitory effect.

Antineoplasic assays

Eagle's minimum essential medium, Earle's balanced salts, non-essential aminoacids (EMEM/neaa), and L-glutamine were purchased from Biosciences, and fetal calf serum (FCS) and Trypsin from Seromed.

In vitro antitumour assays were performed by an adaptation of the method described by Bergeron et al.[34] and the activity screened against the following cell lines: P-388 (ATCC CCL 46; suspension culture of a lymphoid neoplasm from a DBA/2 mouse), A-549 (ATCC CCL 185; monolayer culture of a human lung carcinoma), HT-29 (ATCC HTB-38; monolayer culture of a human colon carcinoma), and MEL-28 (ATCC HTB-72; monolayer culture of a human melanoma). The cells were maintained in logarithmic growth in EMEM/neaa, supplemented with 5% FCS, 10^{-2} M sodium bicarbonate and 0.1 g/L penicillin G/0.1 g/L streptomycin sulfate and placed in 16-mm diameter wells at 1×10^4 (P-388), 2×10^4 (A-549, MEL-28, and HT-29) cells per well, respectively. The cytotoxicity was evaluated by addition of 1 mL aliquots of a solution of the compound in EMEM 5% FCS. A separate set of cultures without the added test compounds was used as a control and to ensure that the cells remained in an exponential phase of growth throughout the test. After three days of incubation at 37 °C in a 10% CO_2 and 98% H_2O atmosphere, the cells were trypsinized and counted in a Coulter Counter ZM. All counts (net cells per well), represent the average of duplicate wells. Comparison of the counts with those of control cultures, allows the calculation of the percentage of growth produced by each compound. The results obtained from cultures treated with different concentrations of each compound, are used to generate dose–response curves from which IC_{50} values were derived.

2-Chlorodimethylaminomethylenamino-3,5-dicyano-6-ethoxy-4-phenylpyridine (6a). A solution of 2-amino-3,5-dicyano-6-ethoxy-4-phenylpyridine (**5a**) (2.64 g, 10 mmol) and *N,N*-dimethyldichloromethylene iminium chloride (1.78 g, 11 mmol) in 1,2-

dichloroethane (25 mL) was heated at reflux for 20 h and then evaporated. The residue was purified by medium-pressure chromatography using hexane:dichloromethane (1:1, v/v) as eluent to yield 2.65 g (75%) of **6a**: mp 145–146 °C. IR (KBr, cm^{-1}): 2220 (CN). ^1H NMR ($CDCl_3$) δ 1.36 (t, 3H, J = 7.1 Hz, CH_3), 3.26 (s, 6H, NMe_2), 4.49 (q, 2H, J = 7.1 Hz, OCH_2), 7.58 (s, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$) δ 14.1 (CH_3), 40.3 (NMe_2), 64.2 (OCH_2), 89.6, 95.6 (C-3, C-5), 114.2, 115.3 (CN), 128.5, 128.7, 130.5, 133.6 (C_6H_4), 143.1 (ClC=N), 160.2, 162.0, 164.8. MS (EI, *m/z*, %): 355 (M^+ + 2, 11), 353 (M^+, 45), 290 (73), 235 (30), 165 (38). ($C_{18}H_{16}N_5OCl$) C, H, N.

Compounds **6b–d** were prepared via the corresponding **5** by a procedure similar to the one shown above. Compound **6b** yield 78%, mp 181–183 °C; **6c** yield 94%, mp 186–188 °C; **6d** yield 90%, mp 146–148 °C.

4-Chloro-6-cyano-2-dimethylamino-7-ethoxy-5-phenylpyrido[2,3-*d*]pyrimidine (7a). A stream of dry hydrogen chloride was passed through a solution of 2-chlorodimethylaminomethylenamino-3,5-dicyano-6-ethoxy-4-phenylpyridine (**6a**) (3.0 g, 8.5 mmol) in 1,2-dichloroethane (50 mL) for 1 h. Stirring was continued at room temperature for four days and the solution was then evaporated. The residue was purified by medium-pressure chromatography using dichloromethane:hexane (3:1, v/v) as an eluent to yield 2.4 g (80%) of **7a**: mp 271–272 °C. IR (KBr, cm^{-1}): 2220 (CN). ^1H NMR ($CDCl_3$) δ 1.48 (t, 3H, J = 7.1 Hz, CH_3), 3.28 (s, 3H, NMe_2), 3.34 (s, 3H, NMe_2), 4.66 (q, 2H, J = 7.1 Hz, OCH_2), 7.31–7.49 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 14.2 (CH_3), 37.3, 37.4 (NMe_2), 64.2 (OCH_2), 96.4 (C-6), 106.6 (C-4a), 114.1 (CN), 128.1, 128.4, 129.5, 135.7 (C_6H_5), 158.4, 160.1, 161.5, 162.6, 165.1. MS (EI, *m/z*, %): 355 (M^+ + 2, 34), 353 (M^+, 100), 338 (21), 325 (31), 310 (43), 296 (56). ($C_{18}H_{16}N_5OCl$) C, H, N.

Compounds **7b–d** were prepared via **5** by a similar procedure. Compound **7b** yield 73%, mp 271–272 °C; **7c** yield 78%, mp 303–305 °C; **7d** yield 76%, mp 258–260 °C.

3,5-Dicyano-6-ethoxy-2-(dimethylamino-4-(4-acetylphenyl)piperazinomethylenamino)-4-phenylpyridine (28). A solution of 2-chlorodimehylaminomethylenamino-3,5-dicyano-6-ethoxy-4-phenylpyridine (**6a**) (0.35 g, 1.0 mmol) and 4′-piperazino-acetophenone (0.25 g, 1.2 mmol) in EtOH (10 mL) was heated at reflux for 36 h and then evaporated. The residue was purified by medium-pressure chromatography using as eluent dichloromethane/hexane (3:1, v/v) to yield 0.33 g (64%) of **28**: mp 221–223 °C. IR (KBr, cm^{-1}): 2220 (CN), 1670 (CO), 1600, 1490, 1400. ^1H NMR ($CDCl_3$) δ 1.40 (t, 3H, J = 7.1 Hz, CH_3), 2.51 (s, 3H, $COCH_3$), 2.97 (s, 6H, NMe_2), 3.40–3.47 (m, 8H, NCH_2), 4.41 (q, 2H, J = 7.1 Hz, OCH_2), 6.84, 7.86 (AA′BB system, 4H, J = 9.0 Hz, C_6H_4), 7.46–7.53 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 14.3 (CH_3), 26.0 ($COCH_3$), 40.1 (NMe_2), 47.1, 47.8 (NCH_2), 63.2 (OCH_2), 85.8, 92.1 (C-3, C-5), 113.9, 115.2, 116.9 (CN), 128.5, 128.6, 130.3, 134.1 (C_6H_5), 153.6, 159.3, 160.7, 162.5, 163.6, 165.1, 165.8, 196.5 (CO). MS (EI, *m/z*, %): 521 (M^+, 1), 360 (22), 347 (100), 290 (37). ($C_{30}H_{31}N_7O_2$) C, H, N.

Compounds **8–27** were prepared as above via the corresponding **6**. Their physicochemical data are shown in Table 1.

3,5-Dicyano-6-(dimethylamino-4-benzylpiperazinomethylenamino)-4-phenylpyridin-2-(1*H*)-one (29). A solution of **10** (1.5 g, 3.0 mmol) and 35% NaOH (2 mL) in ethanol was heated at reflux for 2 h and then evaporated. The residue was diluted with water (40 mL) and the solution adjusted with 2 N HCl to pH 7. The resultant precipitate was filtered and recrystallized from ethanol to yield 1.0 g (71%) of **29**: mp 239–241 °C. IR (KBr, cm^{-1}): 3400, 2220 (CN), 1660 (CO). ^1H NMR ($CDCl_3$) δ 2.53 (br s, 4H, $2NCH_2$), 3.08 (s, 6H, NMe_2), 3.45 (br s, 4H, 2 x NCH_2), 7.31 (s 5H, C_6H_5), 7.51 (s 5H, C_6H_5), 9.07 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 40.5 (NMe_2), 48.7, 52.4 (NCH_2), 62.8 (CH_2Ph), 84.0, 87.6 (C-3, C-5), 116.7, 116.8 (CN), 127.4, 128.1, 128.4, 128.6, 129.1, 130.6, 134.3, 137.2 (C_6H5), 157.3, 162.2, 163.5, 164.3. MS (FAB, *m/z*, %): 466 [$(MH)^+$, 100], 421 (8), 376 (10), 290 (40). ($C_{29}H_{31}N_7O$) C, H, N.

Compound **30** was prepared as above; physicochemical data are shown in Table 1.

3,5-Dicyano-6-ethoxy-2-(dimethylaminomethylaminomethylenamino)-4-phenylpyridine (31). A solution of 2-chlorodimehylaminomethylenamino-3,5-dicyano-6-ethoxy-4-phenylpyridine (**6a**) (0.35 g, 1.0 mmol) and 40% wt methylamine solution in water (0.09 g, 1.2 mmol) in EtOH (10 mL) was heated at reflux for 24 h and then evaporated. The residue was triturated with water and the solid formed was filtered off and recrystallized from EtOH to yield 0.23 g (68%) of **31**: mp 219–220 °C. IR (KBr, cm^{-1}): 2220 (CN). ^1H NMR ($CDCl_3$) δ 1.30 (t, 3H, J = 7.1 Hz, CH_3), 2.90 (d, 3H, J = 4.7 Hz, $NHCH_3$), 3.07 (s, 6H, NMe_2), 4.42 (q 2H, J = 7.1 Hz, OCH_2), 6.52 (q, 1H, J = 4.6 Hz, NH), 7.45–7.73 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 14.3 (CH_2CH_3), 29.9 ($NHCH_3$), 38.2 (NMe_2), 62.6 (OCH_2), 80.9, 89.5 (C-3, C-5), 116.7, 118.0 (CN), 128.3, 129.7, 134.7 (C_6H_5), 160.3, 161.6, 162.8, 165.3. MS (EI, *m/z*, %): 348 (M^+, 56). ($C_{19}H_{20}N_6O$) C, H, N.

Compounds **32** (mp 216–218 °C, 72% yield) and **33** (mp 201–203 °C, 63% yield) were prepared via **6a** by a similar procedure .

3,5-Dicyano-6-ethoxy-2-(3,3-dimethyl-ureido)-4-phenylpyridine (34). A solution of 2-chlorodimethylaminomethylenamino-3,5-dicyano-6-ethoxy-4-phenylpyridine (**6a**) (0.22g, 0.62 mmol) and 25% NH_4OH (0.1 mL) in acetonitrile (5 mL) was stirred at room temperature for 1 h. The solid formed was filtered off and recrystallized from ethanol/acetone to yield 0.16

g (77%) of **34**: mp 238–239 °C. IR (KBr, cm^{-1}): 3450, 3300 (NH), 2220 (CN). ^1H NMR ($CDCl_3$) δ 1.48 (t, 3H, J = 7.1 Hz, CH_3), 3.18 (s, 6H, NMe_2), 4.37 (q, 2H, J = 7.1 Hz, OCH_2), 7.19 (br s, 1H, NH), 7.47–7.57 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 14.2 (CH_3), 37.2 (NMe_2), 63.4 (OCH_2), 84.7, 115.4 (CN), 118.8 (CN), 128.5, 128.6, 130.2, 134.2 (C_6H_5), 157.2, 160.3, 163.7, 164.8. MS (EI, *m/z*, %): 335 (M^+, 20), 334 (100), 319 (46), 305 (42), 291 (81). ($C_{18}H_{17}N_5O_2$) C, H, N.

6-Cyano-2-dimethylamino-6-ethoxy-5-phenyl-4-(4-methylpiperidino)pyrido[2,3-*d*]pyrimidine (35). A solution of 4-chloro-6-cyano-2-dimehylamino-7-ethoxy-5-phenylpyrido[2,3-*d*]pyrimidine (**7a**) (0.35 g, 1.0 mmol) and 4-methylpiperidine (0.12 g, 1.2 mmol) in EtOH (10 mL) was heated at reflux for 36 h. The solid formed was filtered off and recrystallized from EtOH to yield 0.32 g (77%) of **35**: mp 193–195 °C. IR (KBr, cm^{-1}): 2220 (CN). ^1H NMR ($CDCl_3$) δ 0.71 (br s, 3H, $CHCH_3$), 0.90–1.40 (m, 4H, piperidine), 1.46 (t, 3H, J = 7.1 Hz, CH_3), 2.28–2.69 (m, 3H, piperidine), 3.28 (s, 6H, NMe_2), 3.70 (br s, 2H, NCH2), 4.63 (q, 2H, J = 7.1 Hz, OCH_2), 7.45 (s, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 14.4 (CH_3), 21.5 ($CHCH_3$), 30.4 (CH), 32.3, 33.7 (CH_2), 37.1 (NMe_2), 49.2 (NCH_2), 63.4 (OCH_2), 97.8 (C-6), 116.3 (CN), 128.1, 129.6, 129.8, 135.4 (C_6H_5), 155.6, 160.7, 165.4. MS (EI, *m/z*, %): 416 (M^+, 29), 373 (8), 318 (13), 290 (17). ($C_{24}H_{28}N_6O$) C, H, N.

Compounds **36–52** were prepared via **7** by a similar procedure. Their physicochemical data are shown in Table 2.

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