

Antimitotic Agents: Structure-Activity Studies with Some Pyridine Derivatives

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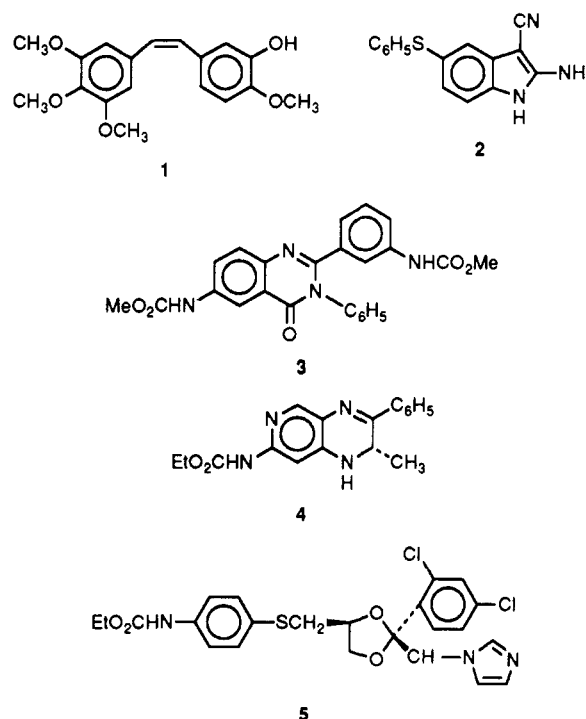
Antitumor activity in mice was observed for the oxime of the previously reported ethyl [6-amino-4-[(1-methyl-2-phenyl-2-oxoethyl)amino]-5-nitropyridin-2-yl]carbamate (8) and several related compounds. These compounds are precursors of the active ethyl pyrido[3,4-*b*]pyrazin-7-ylcarbamates (e.g., 4), which are potent antimitotic agents. In the 5-nitropyridine series overall biological activity was reduced by replacement of the oxime moiety with a keto or alcohol group and by replacement of the 1-methyl group of the side chain with hydrogen. Reduction of the nitro group of the 5-nitropyridines containing an alcohol in the side chain to the corresponding 5-aminopyridines increased biological activity. Preliminary studies showed that the 5-nitropyridine oximes were considerably less potent than the pyridopyrazines as antimitotic agents and that the former are apparently not converted to the latter in vivo. The inhibition of the incorporation of pyrimidine nucleosides into DNA and RNA was identified as another possible mode of action of the 5-nitropyridine oximes.

A number of compounds with diverse structures, combretastatin A-4 (1),¹ amphetamine (2),¹ the 4(3*H*)-quinazolinone 3,² and the 1,2-dihydropyrido[3,4-*b*]pyridine (S)-4,³ are known to compete with colchicine for binding sites on tubulin (Chart I). This mode of action interferes with the polymerization of tubulin to give microtubules, and as a result these agents have shown anticancer activity against experimental tumors in mice. Another microtubule inhibitor, *cis*-tubulazole (5) but not the *trans* isomer, has also shown activity in mice.⁴ In the resynthesis of (R*S*)-4, under the direction of the Drug Synthesis and Chemistry Branch, NCI, a 5-nitropyridine precursor showed activity in mice similar to that observed for the target compound. This result prompted the search for biological activity in previously prepared pyridine intermediates. Another objective was to determine if the pyridine compounds were antimitotic agents and if this activity was a result of metabolic transformation to 1,2-dihydropyrido[3,4-*b*]pyrazines.

Chemistry

In previous work, the synthesis of (1,2-dihydropyrido[3,4-*b*]pyrazin-7-yl)carbamates required the preparation of a variety of 5-nitropyridine intermediates (Scheme I). The alkylation of α -amino ketone oximes with ethyl (6-amino-4-chloro-5-nitropyridin-2-yl)carbamate (6) provided the ketone oximes 7-15.⁵⁻⁹ Compound 29, an analogue of 8, was prepared from 6 and 1-benzimidoyl-1-methyl-

Chart I



hydrazine. Two of the oximes, 7 and 8, were hydrolyzed under acidic conditions to give the corresponding ketones 16⁵ and 17.⁶ In another approach α -amino alcohols were alkylated with 6 to give the 5-nitropyridines (30-40)

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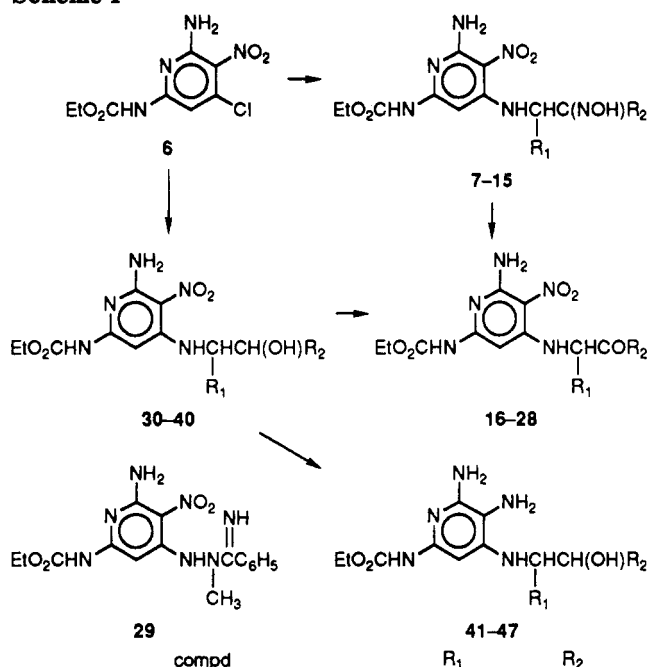
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Scheme I

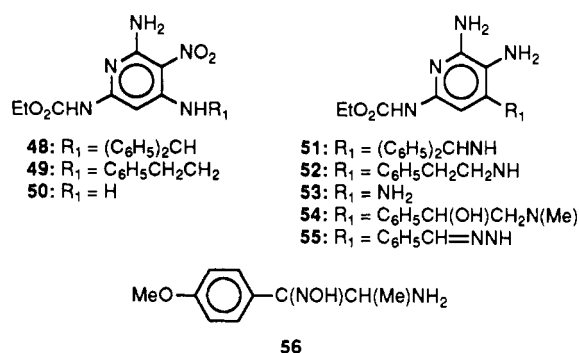


compd	R ₁	R ₂
7, 16	H	C ₆ H ₅
8, 9, ^a (RS)-17	Me	C ₆ H ₅
(S)-18, (R)-19	Me	C ₆ H ₅
(1S,2R)-30, (1R,2S)-31	Me	C ₆ H ₅
(1S,2R)-41, (1R,2S)-42	Me	C ₆ H ₅
(RS)-10, (S)-11 (E-oxime), ^b (S)-12 (Z-oxime) ^b	Me	4-HOC ₆ H ₄
(RS)-20, (S)-21, (R)-22	Me	4-HOC ₆ H ₄
(1SR,2RS)-32, (1S,2R)-33	Me	4-HOC ₆ H ₄
(1R,2S)-34, (1S,2R)-43	Me	4-HOC ₆ H ₄
(1R,2S)-44	Me	4-HOC ₆ H ₄
13	Me	4-MeOC ₆ H ₄
14	Me	3-O ₂ NC ₆ H ₄
15	C ₆ H ₅	C ₆ H ₅
23, 35	Me	3,4-(CH ₂ O ₂)C ₆ H ₃
24, 36, 45	Me	C ₆ H ₅ OCH ₂
25, 37	Et	C ₆ H ₅
26, 38	Me	C ₆ H ₅ CH ₂
27, 39, 46	Me	Bu
28, 40, 47	Bu	Me

^a Methyl carbamate. ^b The isomer in which the side chain CH adjacent to the oxime group has the greater value for the chemical shift was arbitrarily assigned to the (Z)-hydroxypyridine ring isomer.

substituted with a side-chain alcohol group.^{3,8,9} Oxidation of this group with the chromium trioxide-pyridine reagent afforded another series of ketones (18–28).^{3,8,9} In addition, treatment of (S)-21 with hydroxylamine gave the oxime isomers (S)-11 and (S)-12.⁸ Catalytic hydrogenation of the nitro group of 30, 31, 33, 34, 36, 39, and 40 provided a series of 5-aminopyridines (41–47) substituted with a side-chain alcohol group. In addition, methods have been reported for the preparation of the related compounds listed in Chart II.^{8,10–13}

Chart II



Results and Discussion

As an initial screen, the pyridine compounds were evaluated for cytotoxicity against cultured lymphoid leukemia L1210 cells (Table I).¹⁴ The same cell line was used to evaluate several of the more active compounds (IC₅₀ < 26 μM) for antimitotic activity (MI_{0.5}).¹⁴ A number of compounds with IC₅₀ values less than 51 μM were tested for antitumor activity in mice implanted with lymphocytic P388 cells.¹⁵

In early work a large number of ketone oximes (e.g., 7) and the corresponding ketones (e.g., 16) with an unsubstituted α-methylene group were prepared⁵ (Scheme I). None of these compounds exhibited in vivo activity against leukemia P388 on a single-dose schedule. These results were consistent with the inactivity of related 4-aminopyridines, the ethyl (5-nitropyridin-2-yl)carbamates 48–50, and the ethyl (5-aminopyridin-2-yl)carbamates 51–55 (Chart II). Although 51, 53, and 55 gave IC₅₀ values less than 10 μM, none of these compounds exhibited in vivo activity. In later work the 2-methyl-1,2-dihydropyrido[3,4-b]pyrazines (e.g., 4) were shown to be more potent in our screens than the related compounds unsubstituted at the 2-position, which prompted the preparation of additional 2-methyl derivatives. During this period the intermediate ketone oxime 8 was shown to have antitumor activity in mice.¹⁷ The inactivity of both 50 and the α-amino ketone oxime 56 indicated that coupling of the side chain with the pyridine moiety was required for activity.

Although 8 inhibited the proliferation of L1210 cells at concentrations 5 orders of magnitude larger than that needed for 4, 8 showed in vivo activity within 1 order of magnitude. The methyl carbamate 9 and the oximes 10–15 showed similar in vitro activity, and for the compounds tested (9, 10, 11, 13, and 14), in vivo activity as well. Of interest, the oxime (E)-(S)-11 was 3 times more cytotoxic than the (Z)-(S)-12 isomer. In contrast, replacement of the methylene carbon adjacent to the oxime group of 8 with nitrogen gave the inactive hydrazino analogue 29. In addition a mitotic index (MI_{0.5}) could not be calculated

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Table I. Biological Data for Ethyl Pyridin-2-ylcarbamates and Related Compounds

compound (ref)	L1210 IC ₅₀ , μM^a (MI _{0.5} , μM^b)	P388, ^c 10 ⁶ tumor cells implanted, ip	
		dose (mg/kg per inj)	% ILS
colchicine (14, 18)	6.4×10^{-3} (21×10^{-3})		
vincristine (14, 18)	3.4×10^{-3} (11×10^{-3})	2	85
4 (3)	9.4×10^{-5} (14×10^{-5})	0.5	64
8 (6)	32 (>100) ^d	240	80
9 (7)	5.8 (>100) ^d	75	82
10 (8)	18	5	57
11 (8)	6.7 (>30)	2	90 ^e
12 (8)	26 (>100)		
13 (9)	4.2 (>100) ^d	13	84
14 (9)	32 (>100) ^d	100	25
15 (9)	45		
17 (6)	37	300	2
18 (3)	27	400	39
19 (3)	24	200	0
20 (8)	1.2 (6.5) ^f	100	71
21 (8)	24 (>100) ^d		
22 (8)	44		
23 (9)	69		
24 (9)	89		
25 (9)	>100		
26 (9)	10	200	10
27 (9)	54		
28 (9)	31		
29	>100		
30 (3)	7.8	200	53
31 (3)	31	200	0
32 (8)	12		
33 (8)	12	200	20
34 (8)	23	200	0
35 (9)	21		
36 (9)	55		
37 (9)	22		
38 (9)	53		
39 (9)	55		
40 (9)	56		
41	1.3	50	36
42	5.9	200	10
43	0.62 (0.72)	100	58
44	51	200	0
45	21		
46	18	200	10
47	1.7 (3)	200 ^g	45
48 (10)		50 ^h	0
49 (11)	>100 ⁱ		
50 (11)	48 ⁱ	200	0
51 (10)	0.26 ^j	200 ^h	0
52 (11)	75 ⁱ		
53 (11)	10 ⁱ	100	0
54 (6)		50 ^h	4
55 (12)	4.6 (15)	200	3
56 (13)	>100		

^a Micromolar concentration of agent that inhibits the proliferation of cultured lymphoid leukemia L1210 cells to 50% growth during 48 h. ^b Micromolar concentration of agent that causes a mitotic index (number of cells in mitosis divided by total cells) of 0.5 for cultured lymphoid leukemia L1210 cells during an exposure period of 12 h. ^c Lymphocytic leukemia P388; treatment was ip on days 1–5; increase in life span (% ILS) at the highest nontoxic dose tested. ^d MI_{0.5} could not be calculated at the concentrations used to determine the IC₅₀. ^e At a dose of 1 mg/kg, 1/6 45th day survivors. ^f When repeated, MI_{0.5} > 10 μM . ^g Highest dose tested. ^h Leukemia L1210, 48, days 1–9; 51, days 1–15. ⁱ KB cell culture (ref 15). ^j HEP-2 cell culture (ref 16). ^k Single dose on day 1; toxic at 100 and 200 mg/kg per inj.

for 11, 12, and 13 within the concentration range used to determine the IC₅₀. The effect of several of the oximes on the mitosis of cultured L1210 cells is given in Table II.¹⁴ Although 9 and 13 arrested about 25% of the cells in metaphase at 12 h, the effect was considerably less than that observed for 4, which afforded 50% of the cells in metaphase at concentrations comparable to the IC₅₀ value.

Table II. Effect of 5-Nitropyridines on the Mitosis of Cultured L1210 Cells^a

compd	IC ₅₀ , μM	% of cells in metaphase at 12 h					
		0 μM	3 μM	10 μM	30 μM	50 μM	100 μM
8	32	2	2	3	4	14	5
9	5.8	2	3	3	25		14
13	4.2	2	6	26	24		2
14	32	2	4	2	2	6	1

^a 4; IC₅₀, 9.4×10^{-5} μM ; MI_{0.5}, 14×10^{-5} μM .

Table III. Effect of 8 on the Mitosis of L1210 Cells in Vivo

compd	dose, mg/kg	% of cells in metaphase		
		6 h	12 h	24 h
control		0.9	1.0	1.6
8	600	0.9	1.4	0.4
	400	0.6	0.8	1.6
4	7.5	5.3	6.9	3.1
	5.0	9.8	6.1	0.7

Table IV. Effect of 5-Nitropyridines on Macromolecular Synthesis in L1210 Cells in Culture^a

compd	concn (μM)	incorporation as percent of control			
		[³ H]dThd: DNA	[³ H]Urd		[³ H]L-Leu: protein
			DNA	RNA	
8	50	12	17	24	37
	25	25	13	22	101
	10	38	42	55	79
9	25	21*	65*	64*	26
	10	46*	62*	52*	40
	5	52*	89*	86*	79
13	10	44	39	35	82
	5	60	60	47	87
	2.5	78	73	55	69

^a Inhibitor in Me₂SO added to cell suspensions 30 min prior to addition of radiolabeled substrates. Data for the 4-h time period except those indicated by asterisk (3-h time period).

These results suggested that the ketone oximes might be metabolically transformed to 1,2-dihydropyrido[3,4-*b*]pyrazines in vivo. As described in the Experimental Section, no evidence for this conversion was observed when 8 was administered to mice followed by examination of the plasma, liver, and urine for the presence of 4. In addition, mice implanted ip with L1210 cells were treated with a single ip injection of 8, 4, or control as previously described for an analogue of 4.¹⁴ Cells were harvested at 6, 12, and 24 h after treatment and examined for the percentage of cells in metaphase (Table III). The results showed that 4, an inhibitor of the polymerization of tubulin,¹⁸ increased the number of cells in metaphase relative to the control, whereas no increase in metaphase cells was observed for 8.

We next investigated the effect of the 5-nitropyridines on macromolecular synthesis in cultured L1210 cells.¹⁹ Representative results are shown in Table IV. The 5-nitropyridines had a more pronounced effect on DNA and RNA synthesis than on protein synthesis. Interestingly, the incorporation of [³H]thymidine into DNA and of [³H]uridine into DNA and RNA were comparably

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inhibited. Similar results were observed for the incorporation of [^3H]deoxyuridine into DNA. The incorporation of [^{14}C]formate, [^3H]inosine, and [^3H]adenosine into DNA and RNA and the incorporation of [^3H]leucine into protein were inhibited by the 5-nitropyridines to similar extents. The incorporation of [^{14}C]hypoxanthine and [^{14}C]adenine into DNA and RNA was not affected by the 5-nitropyridines. The pattern of inhibition of nucleobase and nucleoside incorporation by the 5-nitropyridines is complex, which suggests multiple sites of action. However, the largest inhibitions were observed for the incorporation of pyrimidine nucleosides into DNA and RNA.

The ketone 17 corresponding to the ketone oxime 8 showed no in vivo activity; however, at a lower dose borderline in vivo activity was showed by the chiral isomer (*S*)-18. Antitumor activity was observed for the ketone 20, which was also one of the most potent compounds in vitro. The IC_{50} values of the remaining ketones suggested that these compounds would give no increase in life span in mice. In addition, the IC_{50} for the ethyl analogue (25) was considerably higher than that observed for the methyl compound 17.

The pyridines containing an alcohol side chain (30–40) were also less active than the ketone oximes with the exception of the chiral isomer (1*S*,2*R*)-30. As mentioned above, the ketone (*S*)-18 derived from this alcohol also showed in vivo activity.

The hydrogenation of selected 5-nitropyridines substituted with alcohol side chains afforded the corresponding, but less stable, 5-aminopyridines 41–47. The correlation of activities in two screens (IC_{50} and % ILS), performed over different time spans, suggested that these compounds were stable under the conditions of the evaluations. Except for 44, these compounds showed greater in vitro activity than the corresponding precursors. In addition 41, 43, and 47 exhibited activity against P388 leukemia in mice. Although in vivo activity was observed with 47 in which the side chain was completely aliphatic, the isomeric compound 46 was inactive. The result with 47 indicated that an aryl moiety in the side chain is not a requirement for antitumor activity. In addition the mitotic index values for 43 and 47 were comparable with the values observed for the IC_{50} . For these compounds the antitumor activity can probably be attributed to the inhibition of mitosis.

In summary, overall activity was greater for the 5-nitropyridine oximes than for the corresponding ketones and alcohols. Apparently the basicity of the pyridine ring is important to activity as indicated by the increase in overall activity in the 5-aminopyridine alcohols relative to the 5-nitropyridine alcohols. Our results suggest that these compounds act by multiple modes of action. The primary mode of action of 8 and similar compounds might be attributed to the inhibition of the incorporation of pyrimidine nucleosides into DNA and RNA, whereas the primary mode of action of 43 and 47 might be attributed to the inhibition of the polymerization of tubulin to microtubules.

Experimental Section

Melting and decomposition temperatures were determined in capillary tubes in a Mel-Temp apparatus. The ^1H NMR spectra were determined on $\text{Me}_2\text{SO}-d_6$ solutions with either a Varian XL-100-15 or a Nicolet NT300NB spectrometer with tetramethylsilane as internal standard. Mass spectra were taken with

a Varian Mat 311A spectrometer operating in either the electron-impact or fast-atom-bombardment mode to provide the M^+ and $(\text{M} + 1)^+$ molecular ion, respectively. The progress of reactions was followed by thin-layer chromatography (TLC) on plates of silica gel from Analtech, Inc. Flash chromatography was performed with silica gel 60 (230–400 mesh) from E. Merck. Tissue extracts were pretreated by passage through a Sep Pak cartridges from Waters Associates. Raney nickel no. 2800 was obtained from Davison Specialty Chemical Co. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within 0.4% of the theoretical value.

Investigation of the Conversion of 8 to (*RS*)-4 in Vivo. Female B6C3F1 mice were given a single ip or iv injection of 8 (35 mg/kg). Control mice received vehicle only. Following dosing, plasma, livers, and urine were collected at selected times through 8 h postdose. After extraction with acetonitrile, samples were analyzed by HPLC using a Spherisorb ODS1 column and a mobile phase of acetonitrile–water (44:56) containing 10 mM ammonium acetate and 0.5 mM triethylamine at a flow rate of 1 mL/min. Detection was by UV absorbance at 365 nm. In this reverse-phase system, the two isomeric forms of 8 eluted with retention times of about 10 and 12 min, and (*RS*)-4 and related deazapteridines after 16 min.

In plasma, 8 was detectable in samples collected following either ip or iv administration. A relatively polar metabolite having a retention time (about 6 min) similar to that of 10 was also resolved. Additionally, a metabolite that eluted at about 23 min was detected. These two products were also detectable in liver.

To investigate the identity of the 23-min metabolite, a liver extract was prepared for analysis by passage through a Sep Pak C18 cartridge. This sample was then cochromatographed with the ketone precursor 17 of (*RS*)-4. Under the HPLC conditions described above, the metabolite had a slightly longer retention time than the ketone, indicating that the two compounds were not identical.

In urine, only traces of 8 were detectable in samples collected for 8 h after ip administration. No products less polar than the parent compound was resolved. Incubation of urine with β -glucuronidase indicated that glucuronides of the parent compound, or its metabolites, were present; however, none of the de-glucuronidated products eluted with a retention time consistent with (*RS*)-4.

Ethyl [6-Amino-4-(2-benzimidoyl-2-methylhydrazino)-5-nitropyridin-2-yl]carbamate (29). To a solution of methyl benzimidate hydrochloride (1.72 g, 10.0 mmol) in MeOH (10 mL) was added methyl hydrazine (0.46 g, 10 mmol). After stirring at room temperature for 2 h, the solution was diluted dropwise with Et_2O (200 mL) over 2 h to deposit crude 1-benzimidoyl-1-methylhydrazine hydrochloride: yield 1.67 g (90%). The ^1H NMR showed the 1-methyl group as a singlet at δ 2.65. A portion of this product (371 mg, 2.00 mmol), 6 (261 mg, 1.00 mmol),²⁰ and Et_3N (303 mg, 3.00 mmol) in 2-propanol (10 mL) were refluxed with stirring for 4 h. The reaction mixture was cooled and diluted with Et_2O (40 mL), and the precipitate was removed by filtration. The filtrate was evaporated to dryness under reduced pressure, the residue was washed with H_2O , and the product was collected by filtration and dried in vacuo over P_2O_5 : yield 320 mg (86%); mp 99–100 °C with formation of a glass and gradual formation of a melt up to 115 °C; MS m/e 374 ($\text{M} + 1$)⁺. The ^1H NMR showed the $\text{N}-\text{CH}_3$ as a singlet at δ 2.96; however, the product was contaminated with a minor amount of an isomeric product in which the N -methyl group appeared at δ 2.93. Anal. ($\text{C}_{16}\text{H}_{19}\text{N}_7\text{O}_4$) C, H, N: calcd, 26.26; found, 25.84.

Ethyl [(1*S*,2*R*)-5,6-Diamino-4-[[2-hydroxy-2-(4-hydroxyphenyl)-1-methylethyl]amino]pyridin-2-yl]carbamate (43). A solution of 33 (2.0 g, 5.1 mmol)⁸ in EtOH (150 mL) was hydrogenated in the presence of Raney nickel (6.0 g, weighed wet, washed three times with H_2O and two times with EtOH) at room temperature and atmospheric pressure for 6 h. The catalyst was removed by filtration (Celite), and the filtrate was evaporated

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to dryness at reduced pressure to give an amber gum. The product was dried in vacuo (P_2O_5) to afford 43 as a glassy amber foam: yield 1.72 g (95%); mp, indefinite with gradual decomposition $>280^\circ\text{C}$; MS m/e 362 ($M + 1$)⁺; ^1H NMR δ 6.62 s (ring CH).²¹ Anal. ($C_{17}H_{23}N_5O_4 \cdot 0.7\text{CH}_3\text{CH}_2\text{OH} \cdot 0.4\text{H}_2\text{O}$) C, H, N.

The following compounds were prepared by a similar method except that 41 required additional Raney nickel (0.6 g) and that 41, 42, and 45–47 after several hours at room temperature were heated at 60°C for 1–6 h to complete the reduction:

Ethyl [(1*S*,2*R*)-5,6-diamino-4-[(2-hydroxy-2-phenyl-1-methylethyl)amino]pyridin-2-yl]carbamate (41) was prepared from 30 (606 mg, 1.61 mmol): yield 511 mg (92%); mp, indefinite, with decomposition $>175^\circ\text{C}$; MS m/e 346 ($M + 1$)⁺; ^1H NMR δ 6.55 s (ring CH).²¹ Anal. ($C_{17}H_{23}N_5O_3 \cdot 0.3\text{CH}_3\text{CH}_2\text{OH} \cdot 0.7\text{H}_2\text{O}$) C, H, N.

Ethyl [(1*R*,2*S*)-5,6-diamino-4-[(2-hydroxy-1-methyl-2-phenylethyl)amino]pyridin-2-yl]carbamate (42) was prepared from 31 (517 mg, 1.38 mmol): yield 442 mg (93%); mp $>300^\circ\text{C}$ with bubbling from 115°C ; MS m/e 346 ($M + 1$)⁺; ^1H NMR, δ 6.39 s (ring CH). Anal. ($C_{17}H_{23}N_5O_3 \cdot 1.4\text{H}_2\text{O}$) C, H, N.

Ethyl [(1*R*,2*S*)-5,6-diamino-4-[[2-hydroxy-2-(4-hydroxyphenyl)-1-methylethyl]amino]pyridin-2-yl]carbamate (44) was prepared from 34 (1.98 g, 5.10 mmol): yield 1.58 g (88%); mp, indefinite, with gradual decomposition $>270^\circ\text{C}$; MS m/e 362 ($M + 1$)⁺; ^1H NMR δ 6.62 s (ring CH).²¹ Anal. ($C_{17}H_{23}N_5O_4 \cdot 0.5\text{CH}_3\text{CH}_2\text{OH} \cdot 0.5\text{H}_2\text{O}$) C, H, N.

Ethyl [(1*R*,2*R*)-5,6-diamino-4-[(2-hydroxy-1-methyl-3-phenoxypropyl)amino]pyridin-2-yl]carbamate (45) was prepared from 36 (212 mg, 0.523 mmol): yield 120 mg (61%); mp $165\text{--}170^\circ\text{C}$ dec; MS m/e 376 ($M + 1$)⁺; ^1H NMR δ 6.56 s (ring CH). Anal. ($C_{18}H_{25}N_5O_4$) C, H, N.

Ethyl [(2*R*,3*R*)-5,6-diamino-4-[(3-hydroxyhept-2-yl)-amino]pyridin-2-yl]carbamate (46) was prepared from 39 (1.02 g, 2.83 mmol): yield 820 mg (89%); mp, indefinite, with gradual decomposition $>70^\circ\text{C}$; MS m/e 326 ($M + 1$)⁺; ^1H NMR δ 6.39 s, 6.41 s (ring CH's of diastereomers). Anal. ($C_{15}H_{27}N_5O_3 \cdot \text{H}_2\text{O}$) C, H, N.

Ethyl [(2*R*,3*R*)-5,6-diamino-4-[(2-hydroxyhept-3-yl)-amino]pyridin-2-yl]carbamate (47) was prepared from 40 (565 mg, 1.55 mmol): yield 471 mg (93%); mp $>300^\circ\text{C}$, with decomposition from 130°C ; MS m/e 326 ($M + 1$)⁺; ^1H NMR δ 6.49 s, 6.51 s (ring CH's of diastereomers).²¹ Anal. ($C_{15}H_{27}N_5O_3 \cdot 0.1\text{CH}_3\text{CH}_2\text{OH} \cdot 0.3\text{H}_2\text{O}$) C, H, N.

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(21) Ethanol was observed (δ 1.06 t) by ^1H NMR in 41, 43, 44, and 47.