Preparation and Structure–Activity Relationships of 4-Substituted Amino-2-methylpyrido[3,4-*d*]pyrimidines as Cytokinin Analogs

Shiro Nishikawa,* Yoshio Nishikimi,† Shinji Maki, Zenzaburo Kumazawa,‡ and Naoki Kashimura

Laboratory of Biofunctional Chemistry, Department of Bioscience, Faculty of Bioresources, Mie University, Tsu, Mie 514, Japan

Various 4-substituted amino derivatives of 2-methylpyrido[3,4-*d*]pyrimidine were prepared to investigate their structure–cytokinin activity relationships. Both an *Amaranthus* betacyanin and a lettuce seed germination bioassay revealed that most anilino and some alkylamino derivatives were active, whereas benzylamino derivatives were inactive, indicating that the new cytokinin analogs differ from N^6-adenines in their substituent effects on the activity. The most active was a *m*-fluoroanilino derivative, which was as potent as N^6-benzyladenine in a tobacco callus bioassay. 2-Alkyl substituents smaller or larger than a methyl group reduced the activity.

Keywords: *Synthetic cytokinin; pyrido[3,4-d]pyrimidine; SAR; lettuce seed germination; betacyanin biosynthesis*

INTRODUCTION

The close similarity in shape of azanaphthalene derivatives to N^6-adenines, which promote cell division of tobacco callus tissues, prompted researchers to develop such derivatives for potential cytokinin activity. In earlier studies, cytokinin analogs of pteridine (Lloyd et al., 1967), quinoline, and quinoxaline (Torigoe et al., 1972) were ineffective or only slightly effective at high concentrations. There was no significant progress in the development of azanaphthalene cytokinins thereafter, although 4-substituted amino-2-methylthiopyrido[2,3-*d*]pyrimidines were shown to have strong anticytokinin activity (Iwamura et al., 1979).

In our research for developing new cytokinin analogs of non-purine type, we studied syntheses and biological activities of a series of pyridopyrimidines and found that 4-(isopentylamino)- and 4-(benzylamino)pyrido[3,4-*d*]-pyrimidine were weakly active and the replacement of the 2-hydrogen with a methyl group increased the activity in an *Amaranthus* betacyanin bioassay (Nishikawa et al., 1986a). Additionally, our later studies revealed that the unsubstituted 4-anilino derivative **1a** exhibited a moderate cytokinin activity in a tobacco callus bioassay (Nishikawa et al., 1994). These results suggested that modification at the 2- and 4-positions of the heteroaromatic ring might further enhance the activity. Importantly, structural optimization at these positions had not been studied previously.

To comprehensively understand the structure–activity relationships of the pyrido[3,4-*d*]pyrimidines, we have prepared various anilino (**1**), benzylamino (**2**), and alkylamino derivatives (**3**) as well as some 2-alkyl-substituted 4-anilino derivatives (**4**) and tested their cytokinin activity in the two bioassays together with a lettuce germination bioassay. As a result, we have found that the *m*-fluoroanilino derivative **1i** is as active as N^6-benzyladenine (BA) in the tobacco callus bioassay.

* Author to whom correspondence should be addressed (e-mail nisikawa@bio.mie-u.ac.jp).

† Present address: Nisso Petrochemical Industries Co., Ltd., 1-3 Kasumi, Yokkaichi, Mie 510, Japan.

‡ Emeritus Professor of Mie University.

This paper describes the synthesis and structure–cytokinin activity relationships of the 4-amino-2-methylpyrido[3,4-*d*]pyrimidines in detail.

MATERIALS AND METHODS

General Methods. Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Hitachi R-22 spectrometer (90 MHz) using tetramethylsilane (TMS) as an internal standard. UV spectra were measured in aqueous solution with a Hitachi UV 200-10, and IR spectra in KBr were taken with a JASCO IR-G spectrophotometer. Microanalyses were performed at the Analytical Center of Kyoto University. The following compounds were synthesized as reported previously: **1a**, **3q**, **3r** (Nishikawa et al., 1986b); **2a**, **3d**, **6a**, **6b**, **7a**, and **7b** (Nishikawa et al., 1986a).

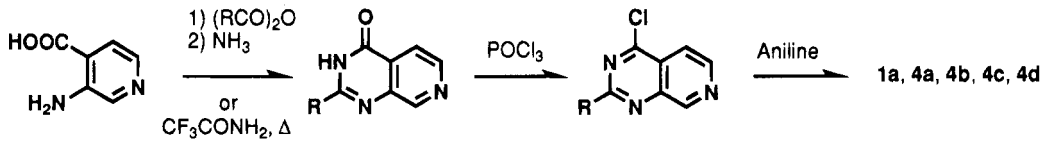
General Procedure for Preparation of 4-Amino-2-methylpyrido[3,4-*d*]pyrimidines. In a manner similar to that for **1a**, a mixture of freshly prepared **7b** (1–2 mmol) and amine (2 equiv) or amine hydrochloride (1 equiv) plus excess triethylamine in ethanol (20–30 mL) was stirred at 40–50 °C for several minutes, dried under reduced pressure, and dissolved in aqueous ethanol to crystallize. The crude product was recrystallized from the same solvent. For compounds **3i–m**, which did not crystallize, the reaction mixture was dried and purified by silica gel chromatography using ethyl acetate–methanol (5:1 v/v) as eluting solvents.

*4-Anilinopyrido[3,4-d]pyrimidine (**4a**).* This compound was prepared from **7a** (Gabriel and Colman, 1902) according to the general procedure: yield, 68%; mp, 215–218 °C; IR 2680 (NH), 1605 (NH), 1570 (aromatic ring) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.2–7.6, 7.85 (m, 5H, Ph), 8.95 (s, 2H, 5-H, 6-H), 9.35 (s, 1H, 8-H); UV λ_{max} (pH 2) 340 (10 200), 288 (5380) nm, λ_{max} (pH 7) 340 (8740), 290 (7820) nm, λ_{max} (pH 11) 340.5 (8450), 290 (7720) nm. Anal. Calcd for $C_{13}H_{10}N_4 \cdot 2H_2O$: C, 60.45; H, 5.46; N, 21.69. Found: C, 60.70; H, 5.42; N, 21.45.

*2-Ethylpyrido[3,4-d]pyrimidin-4(3H)-one (**6c**).* As in the case of **6b**, a mixture of 3-aminoisonicotinic acid (**5**; 3.00 g, 21.7 mmol) and propionic anhydride (14 mL) was refluxed for 2 h. The excess propionic anhydride was removed by distillation under reduced pressure to give crude 2-ethylpyrido[3,4-*d*][1,3]oxazin-4-one, to which was added 15 N ammonia (20 mL), and then the mixture was stirred for 1 h until it became clear. Additional stirring resulted in precipitates, which were filtered and dried. The second crop was obtained from the filtrate: yellowish brown needles (3.03 g, 80%); mp, 307 °C; IR 2980, 2900 (CH), 2800 (NH), 1690 (CONH) cm^{-1}; ^{1}H NMR

0021-8561/95/1443-1034$09.00/0

Scheme 1. Synthesis of 2-Substituted 4-Anilinopyrido[3,4-*d*]pyrimidines



5

6a R=H
6b R=Me
6c R=Et
6d R=i-Pr
6e R=CF_3

7a R=H
7b R=Me
7c R=Et
7d R=i-Pr
7e R=CF_3

(DMSO-d_6) δ 1.25 (t, 3H, J = 8 Hz, CH_3), 2.68 (q, 2H, J = 8 Hz, CH_2), 8.03 (d, 1H, J = 5 Hz, 5-H), 8.61 (d, 1H, J = 5 Hz, 6-H), 9.00 (s, 1H, 8-H), 12.24 (broad, 1H, NH). Anal. Calcd for $C_9H_9N_3O{\cdot}0.1H_2O$: C, 61.08; H, 5.24; N, 23.74. Found: C, 61.20; H, 5.28; N, 23.57.

*4-Chloro-2-ethylpyrido[3,4-d]pyrimidine (**7c**).* In a manner similar to that for **7b**, a mixture of **6c** (500 mg, 2.85 mmol), $POCl_3$ (500 mg, 3.25 mmol), and *N,N*-dimethylaniline (1.00 g, 8.26 mmol) in anhydrous benzene (140 mL) was refluxed for 18 h. The reaction mixture was chromatographed on silica gel in a short column by successive elutions with benzene and benzene–ethyl acetate (5:1 v/v) to afford **7c** as colorless needles (190 mg, 35%): mp, 48–49 °C; IR 2970, 2940 (CH), 1575 (aromatic ring) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.46 (t, 3H, J = 8 Hz, CH_3), 3.18 (q, 2H, J = 8 Hz, CH_2), 8.02 (d, 1H, J = 5 Hz, 5-H), 8.83 (d, 1H, J = 5 Hz, 6-H), 9.51 (s, 1H, 8-H).

*4-Anilino-2-ethylpyrido[3,4-d]pyrimidine (**4b**).* This compound was synthesized from **7c** by amination according to the general procedure: yield, 41%; mp, 184–186 °C; IR 3280 (NH), 3060, 2960, 2930 (CH), 1565 (aromatic ring) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.32 (t, 3H, J = 8 Hz, CH_3), 2.86 (q, 2H, J = 8 Hz, CH_2), 7.19, 7.48, 8.03 (5H, Ph), 8.45 (d, 1H, J = 5 Hz, 5-H), 8.68 (d, 1H, J = 5, 6-H), 9.14 (s, 1H, 8-H), 9.15 (s, 1H, NH); UV λ_{max} (pH 2) 341 (14 200), 285 (6530) nm, λ_{max} (pH 7) 345 (11 500), 291 (10 000) nm, λ_{max} (pH 12) 345 (11 100), 291 (9250) nm. Anal. Calcd for $C_{15}H_{14}N_4{\cdot}0.5H_2O$: C, 69.48; H, 5.83; N, 21.61. Found: C, 69.52; H, 5.75; N, 21.26.

*2-Isopropylpyrido[3,4-d]pyrimidin-4(3H)-one (**6d**).* Similarly, this compound was prepared by the reaction of **5** with isobutyric anhydride, followed by treatment with 15 N ammonia: yield, 79%; mp, 274–277 °C; IR 3030, 2970 (CH), 2850 (NH), 1705 (CONH) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.29 [d, 6H, J = 7 Hz, $C(CH_3)CH_3$], 2.98 (m, 1H, CH), 7.97 (d, 1H, J = 5 Hz, 5-H), 8.65 (d, 1H, J = 5 Hz, 6-H), 9.02 (s, 1H, 8-H), 12.47 (broad, 1H, NH). Anal. Calcd for $C_{10}H_{11}N_3{\cdot}0.1H_2O$: C, 62.88; H, 5.91; N, 22.00. Found: C, 62.67; H, 5.86; N, 21.87.

*4-Chloro-2-isopropylpyrido[3,4-d]pyimidine (**7d**).* Similar dehydroxychlorination of **6d** and subsequent chromatographic separation gave **7d** as syrup: yield, 83%; IR 2970, 2930, 2880 (CH), 1570 (C=N) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.43 [d, 6H, J = 7 Hz, $C(CH_3)CH_3$], 3.39 (m, 1H, J = 7 Hz, CH), 8.01 (d, 1H, J = 5 Hz, 5-H), 8.81 (d, 1H, J = 5 Hz, 6-H), 9.51 (s, 1H, 8-H).

*4-Anilino-2-isopropylpyrido[3,4-d]pyrimidine (**4c**).* Amination according to the general procedure gave **4c**: yield, 66%; mp, 174–176 °C; IR 3280 (NH), 3050, 2960 (CH), 1600 (NH), 1565 (aromatic ring) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.32 [t, 6H, J = 8 Hz, $C(CH_3)CH_3$], 3.09 (m, 1H, J = 8 Hz, CH), 7.20, 7.45, 8.04 (5H, Ph), 8.45 (d, 1H, J = 5 Hz, 5-H), 8.69 (d, 1H, J = 5 Hz, 6-H), 9.14 (s, 1H, 8-H), 9.94 (s, 1H, NH); UV λ_{max} (pH 2) 345 (15 200), 286 (6170) nm, λ_{max} (pH 7) 346.5 (12 500), 292 (9990) nm, λ_{max} (pH 12) 347 (12 100), 292 (9690) nm. Anal. Calcd for $C_{16}H_{16}N_4$: C, 72.70; H, 6.10; N, 21.20. Found: C, 72.37; H, 6.21; N, 21.06.

*2-(Trifluoromethyl)pyrido[3,4-d]pyrimidin-4(3H)-one (**6e**).* In a manner analogous to the method reported (Dominy and Abu El-Haj, 1974; Abu El-Haj and Dominy, 1976), **5** (2.00 g, 14.4 mmol) was heated with trifluoroacetamide (6.55 g, 57.6 mmol) at 210 °C for 5 h and crystallized from aqueous ethanol to yield **6e** (1.68 g, 79%): mp, 278–281 °C (lit. 270–272 °C); IR 3030 (CH), 2750, 1710 (CONH), 1210, 1160 (CF_3) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.06 (d, 1H, J = 5 Hz, 5-H), 8.83 (d, 1H, J = 5 Hz, 6-H), 9.22 (s, 1H, 8-H). Anal. Calcd for $C_8H_4N_3OF_3$: C, 44.66; H, 1.87; N, 19.53. Found: C, 44.22; H, 1.70; N, 19.30.

*4-Chloro-2-(trifluoromethyl)pyrido[3,4-d]pyrimidine (**7e**).* Similar dehydroxychlorination of **6e** with $POCl_3$ followed by chromatographic purification gave **7e** as reddish brown needles: yield, 58%; mp, 102–104 °C; IR 3030 (CH), 1570 (aromatic ring), 1220, 1165 (CF_3) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.16 (d, 1H, J = 5 Hz, 5-H), 9.05 (d, 1H, J = 5, 6-H), 9.97 (s, 1H, 8-H).

*4-Anilino-2-(trifluoromethyl)pyrido[3,4-d]pyrimidine (**4d**).* This compound was prepared according to the general procedure: yield, 90%; mp, 215–216 °C; IR 3390 (NH), 3030 (CH), 1570 (aromatic ring), 1205 (CF_3) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.28, 7.53, 7.95 (5H, Ph), 8.59 (d, 1H, J = 5 Hz, 5-H), 8.90 (d, 1H, J = 5 Hz, 6-H), 9.35 (s, 1H, 8-H), 10.59 (s, 1H, NH); UV λ_{max} (pH 2) 342.5 (13 500), 300^s (9210) nm, λ_{max} (pH 7) 341 (13 200), 300^s (8990) nm, λ_{max} (pH 11) 341 (13 700), 300^s (9520) nm. Anal. Calcd for $C_{14}H_9N_4F_3$: C, 57.97; H, 3.13; N, 19.30. Found: C, 58.22; H, 3.11; N, 19.24.

*4-(m-Fluoroanilino)pyrido[3,4-d]pyrimidine (**4e**).* Similar amination of **7a** with *m*-fluoroaniline gave **4e**: yield, 80%; mp, 202–203 °C; IR 3270 (NH), 3020 (CH), 1575 (aromatic ring) cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.9–8.1 (m, 4H, Ph), 8.52 (d, 1H, J = 5 Hz, 5-H), 8.79 (d, 1H, J = 5 Hz, 6-H), 9.26 (s, 1H, 8-H). Anal. Calcd for $C_{13}H_9N_4F{\cdot}1H_2O$: C, 60.46; H, 4.29; N, 21.69. Found: C, 60.51; H, 4.06; N, 21.51.

Determination of Biological Activity. *Amaranthus Betacyanin Bioassay.* According to the reported method (Biddington and Thomas, 1973), derooted seedlings grown for 60 h at 28 °C in the dark were incubated in a solution containing the sample and tyrosine in a phosphate buffer (pH 6.3) for 20–24 h under the conditions reported previously (Koyama et al., 1985).

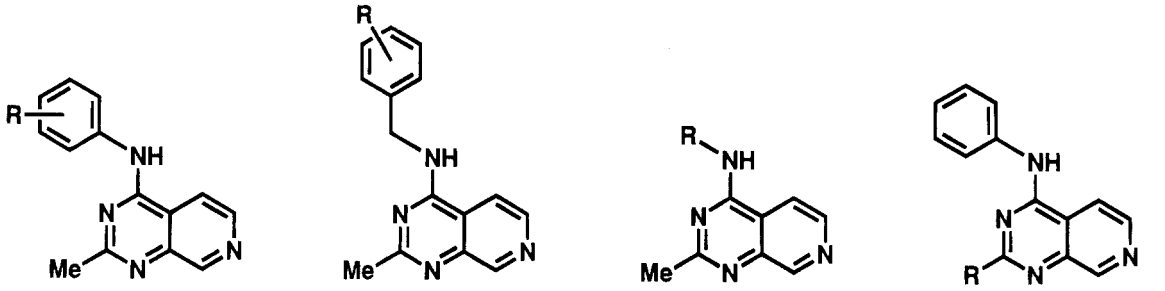
Lettuce Seed Germination Bioassay. As reported previously (Koyama et al., 1985), seeds of *Lactuca sativa* cv. Great Lakes 366 were grown on filter paper moistened with an aqueous solution of the sample at 30–31 °C for 4 days in the dark.

Tobacco Callus Bioassay. This bioassay method has been reported (Nishikawa et al., 1986b). Callus tissues derived from *Nicotiana tabacum* var. Wisconsin No. 38 were grown on an agar medium (Linsmaier and Skoog, 1965) at 28 °C for 40 days.

RESULTS AND DISCUSSION

Synthesis of 4-Aminopyrido[3,4-*d*]pyrimidines. Amination of the 4-chloride **6b** with different amines in EtOH under mild conditions proceeded smoothly, giving the 4-anilino **1a–p**, 4-benzylamino **2a–m**, and 4-alkylamino derivatives **3a–r** (Figure 1). Most compounds synthesized were obtained as hydrates after crystallization from aqueous EtOH (Table 1). While the anilino derivatives **1** were fluorescence-inactive, the benzylamino derivatives **2** and alkylamino derivatives **3** were strongly fluorescent, when irradiated at 254 nm.

2-Alkyl-substituted 4-anilino derivatives **4b–d** were prepared from 3-aminoisonicotinic acid (**5**) via pyrido[3,4-*d*]pyrimidin-4(3H)-ones **6c–e**. Cyclization of **5** with propionic anhydride and isobutyric anhydride, followed by treatment with ammonia (Gelling and Wibberley, 1969), yielded the 4(3H)-ones **6c** and **6d**, respectively (Scheme 1). Since similar cyclization using trifluoroacetic anhydride (Dominy and Abu El-Haj, 1974) to obtain **6e** was unsuccessful, it was prepared through



1a	R=H	2a	R=H	3a	R=n-Bu	4a	R=H
1b	R=*o*-Me	2b	R=*o*-Me	3b	R=i-Bu	4b	R=Et
1c	R=*m*-Me	2c	R=*m*-Me	3c	R=n-Pen	4c	R=i-Pr
1d	R=*p*-Me	2d	R=*p*-Me	3d	R=i-Pen	4d	R=CF_3
1e	R=*o*-OMe	2e	R=*o*-OMe	3e	R=n-Hex	4e	R=H (*m*-F)
1f	R=*m*-OMe	2f	R=*m*-OMe	3f	R=i-Hex		
1g	R=*p*-OMe	2g	R=*p*-OMe	3g	R=c-Pen		
1h	R=*o*-F	2h	R=*o*-F	3h	R=c-Hex		
1i	R=*m*-F	2i	R=*m*-F	3i	R=$(CH_2)_4OH$		
1j	R=*p*-F	2j	R=*p*-F	3j	R=$(CH_2)_5OH$		
1k	R=*o*-Cl	2k	R=*o*-Cl	3k	R=$(CH_2)_3OMe$		
1l	R=*m*-Cl	2l	R=*m*-Cl	3l	R=$(CH_2)_3OEt$		
1m	R=*p*-Cl	2m	R=*p*-Cl	3m	R=$(CH_2)_3O$-n-Pr		
1n	R=*o*-OH			3n	R=Δ^2-Isopentenyl		
1o	R=*m*-OH			3o	R=(*E*)-4-Hydroxy-3-methyl-2-butenyl		
1p	R=*p*-OH			3p	R=Furfuryl		
				3q	R=$(CH_2)_2Ph$		
				3r	R=$(CH_2)_3Ph$		

Figure 1. Structures of 4-substituted aminopyrido[3,4-*d*]pyrimidines.

an alternative cyclization of **5** with trifluoroacetamide at high temperature (Gabriel and Colman, 1902) in good yield. Dehydroxychlorination of **6c–e** afforded the 4-chlorides **7c–e**, which were unstable and used for subsequent amination immediately after chromatographic separation. Amination of **7a** and **7c–e** with aniline afforded the 2-unsubstituted **4a** and 2-substituted 4-anilino derivatives **4b–d**.

Biological Activities. In an *Amaranthus* betacyanin bioassay, most anilino derivatives **1** and some alkylamino derivatives **3** were active, whereas none of the benzylamino derivatives **2** except for the unsubstituted **2a** was active (Table 2). Substitution of a hydrogen on the phenyl ring in the benzyl group of **2a** with any group resulted in a complete loss of activity. This is in marked contrast to the results obtained with N^6-adenine cytokinins; most N^6-benzyladenines substituted with a small group on their phenyl ring exhibit more or less cytokinin activity in this bioassay (Nishikawa, unpublished result).

Among the anilino derivatives **1**, methyl-, fluoro- and chloro-substituted derivatives showed the activity order of *m*- (**1c**, **1i**, and **1l**) > *o*- (**1b**, **1h**, and **1k**) > *p*-isomers (**1d**, **1j**, and **1m**). For methoxy and hydroxy derivatives, the activity order of *o*- and *m*-isomers was reversed, and thus *o*- (**1e** and **1n**) > *m*- (**1f** and **1o**) ≫ *p*-isomers (**1g** and **1p**); the *p*-isomers were inactive. Compounds **1c** and **1l** were almost as active as unsubstituted **1a**. Noteworthy is the effect of meta substitution with a fluoride atom on the activity. Compound **1i** was the most potent among the pyrido[3,4-*d*]pyrimidines tested. Meta substitution with a fluorine atom on the phenyl ring resulted in a 4-fold increase in the activity compared to unsubstituted **1a**. Compound **1i** was about one-fifth as active as BA in this bioassay. A similar increase in the activity was observed for 4-(*m*-fluoroanilino)pyrido[3,4-*d*]pyrimidine, the 2-unsubstituted counterpart **4e**, which was 5 times more active than the parent compound **7a**.

Among the alkyl derivatives **3**, ω-hydroxyalkylamino derivatives **3i** and **3j** and ω-alkoxyalkylamino derivatives **3k–m**, both of which have a hydrophilic substituent, were inactive. In addition, compound **3o**, which has the same substituent as *trans*-zeatin, a naturally occurring potent cytokinin, was also inactive. On the other hand, some alkylamino derivatives having a hydrophobic substituent showed cytokinin activity, although their activities were low compared to that of the substituted anilino derivatives **1**. These results indicate that hydrophilic 4-substituents disfavor the activity of the pyrido[3,4-*d*]pyrimidines. Among the active alkylamino derivatives, compounds **3g** and **3n** were more active than the others, suggesting that their higher activity is ascribed to the planarity of the 4-substituent with an appropriate size. The importance of the planarity of the substituents of cytokinin analogs has been shown for *N*-phenyl-*N'*-(4-pyridyl)ureas (Yamaguchi and Shudo, 1992) and 6-vinylpurines (Nishikawa et al., 1986c).

Although kinetin analog **3p** was less active than 2iP analog **3n**, probably because of the less hydrophobic nature of its 4-substituent, it was more active than BA analog **2a**. The weak activity of **2a** is attributed to the "zigzag" activity of the aralkyl derivatives of cytokinins involving 4-aminopyrido[3,4-*d*]pyrimidine (Nishikawa et al., 1986b). Thus, the activity order of the BA, 2iP, *trans*-zeatin, and kinetin analogs was **3n** > **3p** > **2a** ≫ **3o** (inactive). On the other hand, the activities of BA, 2iP, *trans*-zeatin, and kinetin in this bioassay were 0.10, 0.28, 0.60, and 2.8 μM, respectively, in terms of a defined concentration, $C_{0.1\mu M\ BA}$ (see footnote in Table 2), and thus the activity order of the N^6-adenines was BA > 2iP > *trans*-zeatin > kinetin. Comparison of the activity order between the pyrido[3,4-*d*]pyrimidines and the N^6-

Table 1. Physical Data of 4-Substituted Amino-2-methylpyrido[3,4-*d*]pyrimidines

compd	yield, %	mp, °C	formula
1b	90	183–184	$C_{15}H_{14}N_4$
1c	84	164–165	$C_{15}H_{14}N_4 \cdot 1.33H_2O$
1d	98	207–209	$C_{15}H_{14}N_4 \cdot 0.75H_2O$
1e	43	156–157	$C_{15}H_{14}N_4O \cdot 1.25H_2O$
1f	86	174–175	$C_{15}H_{14}N_4O \cdot 1.75H_2O$
1g	46	185–187	$C_{15}H_{14}N_4O \cdot 2H_2O$
1h	45	205–206	$C_{14}H_{11}N_4F$
1i	40	221–222	$C_{14}H_{11}N_4F \cdot 1.75H_2O$
1j	98	245–246	$C_{14}H_{11}N_4F \cdot 1.75H_2O$
1k	48	174–175	$C_{14}H_{11}N_4Cl$
1l	94	189–190	$C_{14}H_{11}N_4Cl \cdot 0.75H_2O$
1m	98	245–247	$C_{14}H_{11}N_4Cl \cdot 2H_2O$
1n	38	245–247	$C_{14}H_{12}N_4O \cdot 1H_2O$
1o	42	287–289	$C_{14}H_{12}N_4O \cdot 1.67H_2O$
1p	15	252–257	$C_{14}H_{12}N_4O \cdot 2H_2O$
2b	83	227–229	$C_{16}H_{16}N_4$
2c	60	147–148	$C_{16}H_{16}N_4 \cdot 0.25H_2O$
2d	93	183–184	$C_{16}H_{16}N_4 \cdot 1.25H_2O$
2e	71	178–179	$C_{16}H_{16}N_4O \cdot 1H_2O$
2f	83	148–149	$C_{16}H_{16}N_4O \cdot 1.67H_2O$
2g	98	150–151	$C_{16}H_{16}N_4O \cdot 0.5H_2O$
2h	81	171–172	$C_{15}H_{13}N_4F$
2i	68	157–159	$C_{15}H_{13}N_4F \cdot 0.75H_2O$
2j	94	178–179	$C_{15}H_{13}N_4F \cdot 2H_2O$
2k	41	194–195	$C_{15}H_{13}N_4Cl$
2l	75	166–167	$C_{15}H_{13}N_4Cl \cdot 2H_2O$
2m	93	210–212	$C_{15}H_{13}N_4Cl \cdot 1.75H_2O$
3a	99	112–113	$C_{12}H_{16}N_4 \cdot 2H_2O$
3b	96	128–130	$C_{12}H_{16}N_4 \cdot 2.2H_2O$
3c	100	114–115	$C_{13}H_{18}N_4 \cdot 2H_2O$
3e	97	96–98	$C_{14}H_{20}N_4 \cdot 2H_2O$
3f	97	96–97	$C_{14}H_{20}N_4 \cdot 1H_2O$
3g	85	125–126	$C_{13}H_{16}N_4 \cdot 2H_2O$
3h	99	175–176	$C_{14}H_{18}N_4 \cdot 1H_2O$
3i	71	171–173	$C_{12}H_{16}N_4O \cdot 0.2H_2O$
3j	75	164–166	$C_{13}H_{18}N_4O$
3k	81	103–104	$C_{12}H_{16}N_4O \cdot 0.75H_2O$
3l	59	92–93	$C_{13}H_{18}N_4O \cdot 1.5H_2O$
3m	53	93–94	$C_{14}H_{20}N_4O \cdot 0.2H_2O$
3n	43	124–126	$C_{13}H_{16}N_4 \cdot 2H_2O$
3o	69	194–195	$C_{13}H_{16}N_4O \cdot 2H_2O$
3p	95	177–179	$C_{13}H_{16}N_4O$

Table 2. Cytokinin Activity of 4-Substituted Aminopyrido[3,4-*d*]pyrimidines Tested in *Amaranthus* Betacyanin Bioassay

compd[a]	$C_{0.1\mu M\ BA}$[b] (μM)	compd[a]	$C_{0.1\mu M\ BA}$ (μM)
1a	2.5	**2a**	380
1b	87	**3a**	190
1c	3.1	**3c**	130
1d	280	**3d**	240
1e	7.1	**3f**	300
1f	11	**3g**	55
1h	6.0	**3o**	24
1i	0.72	**3q**	210
1j	7.6	**3r**	90
1k	25	**4a**	36
1l	2.0	**4b**	6.2
1m	150	**4c**	76
1n	5.4	**4e**	7.2
1o	83		

[a] Inactive: **1g**, **1p**, **2b–m**, **3b**, **3e**, **3h–m**, **3o**, **3r**, and **4d**. [b] Concentration at which sample gives the same amount of betacyanin as that induced by 0.1 μM BA.

adenines clearly shows that they differ in their substituent effects on cytokinin activity.

A lettuce seed germination bioassay gave similar results (Table 3). Most anilino derivatives **1** were active, whereas none of the benzylamino derivatives **2** was active. For the alkylamino derivatives **3**, only compounds **3g**, **3n**, and **3p** were weakly active, **3g** and **3n** being stronger than the kinetin analog **3p**. Among the anilino derivatives **1**, *m*-fluoroanilino derivative **1i** was approximately 3 times more active than the unsubstituted **1a**, and **1h** was as active as the latter. Anilino derivatives other than **1i** and **1h** were less active than **1a**. The activity order of the anilino derivatives **1** was *m*- > *o*- ≫ *p*-isomers, except for methoxy derivatives for which the *o*- and *m*-isomers were equally active. The general trend of the activity order was almost identical with that in the *Amaranthus* betacyanin bioassay. Again, compound **1i** was the most active among the compounds tested.

Table 3. Cytokinin Activity of 4-Substituted Amino-2-methylpyrido[3,4-*d*]pyrimidines Tested in a Lettuce Seed Germination Bioassay

compd[a]	C_{50}[b] (μM)	compd[a]	C_{50}[b] (μM)
1a	0.81	**1j**	9.8
1b	9.3	**1k**	15
1c	1.3	**1l**	2.8
1d	120	**1o**	1.6
1e	7.6	**3g**	27
1f	6.9	**3n**	19
1h	0.56	**3p**	240
1i	0.23		

[a] Inactive: **1g**, **1m**, **1n**, **1p**, **2a–m**, **3a–f**, **3h–m**, **3o**, and **3q–r**. [b] Concentration at which sample gives 50% germination after 4 days in the dark.

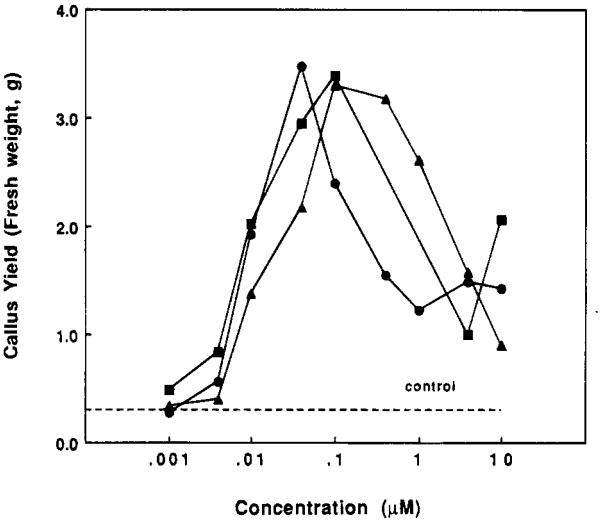


Figure 2. Effects of 4-(*m*-fluoroanilino)-2-methylpyrido[3,4-*d*]pyrimidine (**1i**), kinetin, and N^6-benzyladenine on the growth of callus tissues of *N. tabacum* var. Wisconsin No. 38: **1i**, ●; kinetin, ▲; N^6-benzyladenine, ■.

To assess the effect of the 2-substituent on cytokinin activity, $C_{0.1\mu M\ BA}$ values of 2-unsubstituted **4a** and 2-alkyl-substituted 4-anilino derivatives **1a**, **4b**, and **4d** were determined to be 36, 2.5, 6.2, and 76 μM, respectively, and compound **4c** was inactive in the *Amaranthus* betacyanin bioassay. Therefore, the activity order for the 2-substituent was Me > Et > H > CF_3 ≫ i-Pr (inactive). When the 2-substituent was methyl, the activity was optimal; alkyl groups smaller or larger than a methyl group diminished the activity.

Tobacco callus bioassay is one of the frequently used standard bioassays for cytokinins. Accordingly, the activity of **1i**, which was the most active in both bioassays, was evaluated in a tobacco callus bioassay for comparison with kinetin and BA. Compound **1i** induced the same maximal callus yield as kinetin and BA (Figure 2). It was more active than kinetin and as active as BA. The enhanced relative activity to BA of **1i** in this bioassay may be attributed to its resistance to metabolism by tobacco callus tissues compared to BA.

In conclusion, optimization of the 2- and 4-substituents of the pyrido[3,4-*d*]pyrimidines led to the finding of a new derivative with high cytokinin activity for practical use. Substituent effects of the cytokinin analogs on the activity were very different from those of N^6-adenines.

Supplementary Material Available: Tables of ^{1}H NMR, UV, IR, and analytical data for 4-substituted amino-2-methylpyrido[3,4-*d*]pyrimidines (7 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

Abu El-Haj, M. J.; Dominy, B. W. 2-Aminonicotinonitrile. U.S. Pat. 3,962,264, 1976; *Chem. Abstr.* **1976**, *85*, 123967h.

Biddington, N. L.; Thomas, T. H. A modified *Amaranthus* Betacyanin Assay for the Rapid Determination of Cytokinins in Plant Extracts. *Planta* **1973**, *111*, 183–186.

Dominy, B. W.; Abu El-Hay, M. J. 2-Substituted Pyrido[2,3-*d*], Pyrido[3,4-*d*], and Pyrido[4,3-*d*]pyrimidine-4(3H)ones, and Herbicidal Compositions Containing Them. Ger. Offen. 2,348,111, 1974; *Chem. Abstr.* **1974**, *81*, 37571k.

Gabriel, S.; Colman, J. Abkommlinge der Chinchomeronsaure II. *Ber. Dtsch. Chem. Ges.* **1902**, *35*, 2821–2852.

Gelling, I. R.; Wibbery, D. G. Pyridopyrimidines V. Synthesis and Properties of Pyrido[3,4-*d*]pyrimidine-4(3H)-ones and -Pyrimidine-2,4(1H, 3H)-diones. *J. Chem. Soc.* **1969**, 931–934.

Iwamura, H.; Murakami, S.; Koga, J.; Matsubara, S.; Koshimizu, K. Quantitative Analysis of Anticytokinin Activity of 4-Substituted-2-methylthiopyrido[2,3-*d*]pyrimidines. *Phytochemistry* **1979**, *18*, 1265–1268.

Koyama, S.; Kumazawa, Z.; Kashimura, N.; Nishida, R. Photochemical Synthesis and Biological Activity of 6-*cis*-Styrylpurine. *Agric. Biol. Chem.* **1985**, *49*, 1859–1861.

Linsmaier, E. M.; Skoog, F. Organic *Growth Factor Requirements* of Tobacco Tissue Cultures. *Planta* **1965**, *18*, 100–127.

Lloyd, R. F.; Skinner, C. G.; Shive, W. 4-Substituted Pteridines, Analogs of Kinetin. *Can. J. Chem.* **1967**, *45*, 2213–2216.

Nishikawa, S.; Kumazawa, Z.; Kashimura, N.; Maki, S.; Nishikimi, Y. Synthesis and Cytokinin Activity of 4- and 2,4-Substituted Pyrido[3,4-*d*]pyrimidine. *Agric. Biol. Chem.* **1986a**, *50*, 495–497.

Nishikawa, S.; Kumazawa, Z.; Kashimura, N.; Nishikimi, Y.; Uemura, S. Alternating Dependency of Cytokinin Activity on the Number of Methylene Units in ω-Phenylalkyl Derivatives of Some Purine Cytokinins and 4-Substituted Pyrido[3,4-*d*]pyrimidines. *Agric. Biol. Chem.* **1986b**, *50*, 2243–2249.

Nishikawa, S.; Kumazawa, Z.; Mizutani, H.; Kashimura, N. Substituent-directing Effects on Cytokinin Activity of the α-Double Bond in the 6-Substituent of Purines. *Agric. Biol. Chem.* **1986c**, *50*, 1089–1091.

Nishikawa, S.; Maki, S.; Nishikimi, Y.; Kumazawa, Z.; Kashimura, N. Cytokinin Activity of 4-Aminopyridopyrimidines toward the Growth of Tobacco Callus. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1709–1710.

Torigoe, Y.; Akiyama, M.; Hirobe, T.; Okamoto, T.; Isogai, Y. Cytokinin Activity of Azaindene, Azanaphthalene, Naphthalene and Indole Derivatives. *Phytochemistry* **1972**, *11*, 1623–1630.

Yamaguchi, K.; Shudo, K. Crystallographic Studies on Urea Cytokinins. *J. Agric. Food Chem.* **1991**, *39*, 793–796.

Received for review October 7, 1994. Accepted January 26, 1995.[⊗]

JF940567X

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1995.