Synthesis and Cytokinin Activity of Fluorescent 7-Phenylethynylimidazo[4,5-*b*]pyridine and Its Riboside

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7-Phenylethynylimidazo[4,5-*b*]pyridine and its riboside have been newly developed as fluorescent carbon-substituted cytokinin analogues. Palladium-catalyzed coupling of 7-iodo-3-(tri-*O*-acetyl-*β*-D-ribofuranosyl)imidazo[4,5-*b*]pyridine with phenylacetylene followed by ammonolysis afforded the 7-phenylethynyl riboside via its tri-*O*-acetate. Acid hydrolysis of the riboside provided its free base, which showed a marked enhancement in fluorescence intensity in an aqueous alkaline solution. The free base and its riboside were more active than the corresponding 6-phenylethynylpurine and its riboside, respectively, in *Amaranthus* betacyanin and tobacco callus bioassays. Surprisingly, the imidazo[4,5-*b*]pyridine base exhibited strong cytokinin activity comparable to that of N^6-benzyladenine in the tobacco callus bioassay. This compound would be useful for studying localization and transport of cytokinins in cells or tissues of plants.

Keywords: *7-Alkynylimidazo[4,5-b]pyridine; 6-alkynyl-1-deazapurine; fluorescent cytokinin analogue; tobacco callus growth; betacyanin biosynthesis*

INTRODUCTION

N^6-Adenines including N^6-isopentenyladenine (2iP) and N^6-benzyladenine (BA, **1**) markedly promote cell division and regulate development of plants. The adenines substantially show no fluorescence, although they have a potent cytokinin activity. Therefore, fluorescent cytokinin analogues with a potent activity have been desired to be accessible, since they would provide a useful tool to study the localization and the transport of cytokinins in plant cells or tissues as well as in binding experiments. In an early study of fluorescent cytokinins, 2iP and BA analogues of benzoadenines, i.e., imidazo[4,5-*g*]- (**2**) and imidazo[4,5-*f*]quinazoline (**3**) (Figure 1) were synthesized for assessing spatial parameters of the central moiety in cytokinins (Specker et al., 1976). However, their cytokinin activities were weak or negligible in a tobacco callus bioassay, although they were fluorescent. The ring expansion of the purine ring to make them fluorescent by inserting a phenyl group resulted in a serious decrease in cytokinin activity. No attention has been paid to fluorescent cytokinins thereafter.

On the other hand, 6-alkenyl- and 6-alkynylpurines were also found to be cytokinin-active (Brathe et al., 1999). We synthesized carbon-substituted analogues of purine (Koyama et al., 1983; Nishikawa et al., 1994), pyrimidine (Nishikawa et al., 1989), and pyridine (Nishikawa et al., 1996) to identify the minimal structure as well as active conformation responsible for the high activity of cytokinins. Such analogues including *β*-substituted styryl derivatives were made conveniently from the corresponding alkynylated azaheterocycles, among which 6-phenylethynylpurine (**5**) exhibited moderate activity, although its riboside **4** (Figure 1) was weak in an *Amarathus* bioassay (Koyama et al., 1982). We noticed that most of the alkynylated azaheterocycles showed fluorescence during the synthetic studies.

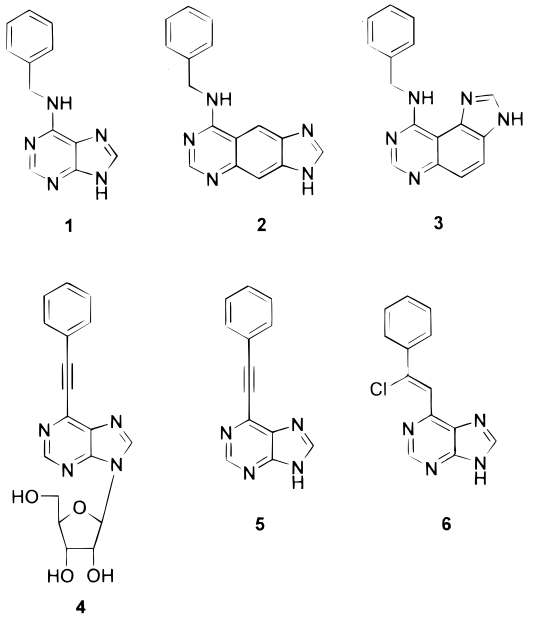

Figure 1. Structures of fluorescent cytokinin analogues and related compounds.

In recent years, purine-related 7-aminoimidazo[4,5-*b*]pyridines and their ribosides have generated considerable interest in their biological activities involving antitumor activity, inhibition of adenosine deaminase (Cristalli et al., 1991), and antiviral activity against human immunodeficiency virus (Cristalli et al., 1995). The structural similarity of N^6-adenines and 7-aminoimidazo[4,5-*b*]pyridines suggested to us the development of new carbon-substituted derivatives of the latter as cytokinin analogues. However, such carbon-substituted

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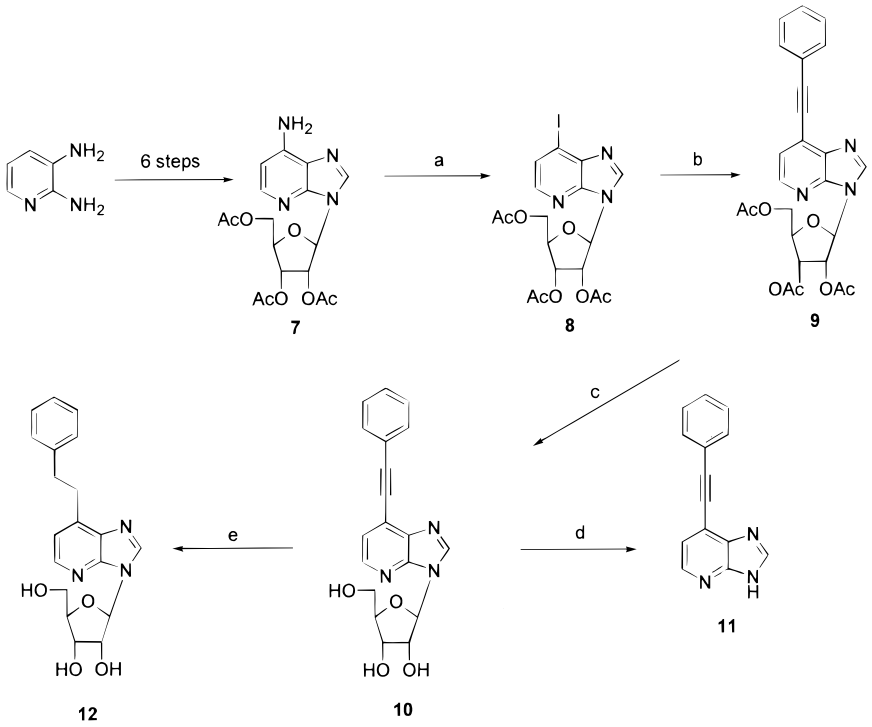
Scheme 1. Synthesis of Alkynyl Derivatives of Imidazo[4,5-*b*]pyridines[a]



[a] Conditions and reagents: (a) CH_2I_2, isoamyl nitrite/MeCN, *hν*, reflux; (b) PhCCH, $(PPh_3)_2PdCl_2$, CuI, Et_3N/MeCN, reflux; (c) NH_3/MeOH; (d) 0.1 N HCl/aqueous dioxane, reflux; (e) H_2, Pd-C/MeOH.

derivatives have been rarely known until now. Early studies of N^6-alkyl (De Roos and Salemink, 1971; Rogozinska et al., 1973; Kroon et al., 1974; Kitano et al., 1975) and N^6-acyl (Sugiyama et al., 1975; Matsubara et al., 1978) derivatives of deazaadenines revealed that, among the deazapurine isomers, only imidazo[4,5-*b*]-pyridines retained high activity. Thus, in the development of new fluorescent cytokinin analogues, we synthesized 7-phenylethynylimizado[4,5-*b*]pyridine (**11**) and its riboside **10** by a rational drug design in which they could be endowed with both fluorescence and potent cytokinin activity by transformation without ring expansion. Finally, we found that the free base **11** showed strong fluorescence in an aqueous alkaline solution and exhibited strong cytokinin activity comparable to that of BA in a tobacco callus bioassay.

RESULTS AND DISCUSSION

Synthesis. Practically, fluorescent cytokinins should meet requirements for both high cytokinin activity and strong fluorescence. Since cytokinins are small molecules, alteration in structure by the introduction of a substituent or a fluorophore of even a relatively small size may diminish or abort its activity, as verified by quantitative structure−activity study of cytokinin-active adenines (Iwamura et al., 1980) as well as our study on the minimal structure of highly active cytokinins (Nishikawa et al., 1996). Taking this into consideration, we followed a different approach in which structural changes in both the side chain and the central moiety were minimized. In our synthesis, the ribosyl group introduced at the 3-position of imidazo[4,5-*b*]pyridine not only constitutes a ribose moiety of the riboside **10** but also functions to increase the reactivity in the palladium-catalyzed coupling and is easily removed by acid hydrolysis in the final step.

Thus, 7-amino-3-(2′,3′,5′-tri-*O*-acetyl-*β*-D-ribofuranosyl)imidazo[4,5-*b*]pyridine (**7**) prepared from 2,3-diaminopyridine was subjected to radical iodination upon irradiation (Nair et al., 1980) (Scheme 1). The reaction of the amino derivative **7** with a mixture of CH_2I_2 and isoamyl nitrite provided 7-iodide **8** in a moderate yield, the structure being confirmed by LC−MS. The coupling of **8** with phenylacetylene to make a conjugated system ensures fluorescence of **10** and **11**. The palladium-catalyzed coupling of **8** with the terminal alkyne (Sonogashira et al., 1975) proceeded smoothly in MeCN (Koyama et al., 1982; Matsuda et al., 1985) to afford 7-alkynylated tri-*O*-acetate **9** in a high yield. Ammonolysis of **9** provided 7-alkynyl riboside **10**, which was further hydrogenated to give phenylethyl compound **12**. The ribosyl group of **10** was cleaved in an aqueous dioxane containing 0.1 N HCl to give its free base **11** in a moderate yield without affecting the triple bond (Koyama et al., 1982; Matsuda et al., 1992). The new imidazo[4,5-*b*]pyridine **11** was resistant to hydrochloride addition, in contrast to the corresponding purine base **5**, which easily undergoes similar hydrogen halide additions under electrophilic conditions to give *β*-substituted 6-styrylpurines (Koyama et al., 1983; Nishikawa et al., 1985).

All the alkynyl derivatives synthesized were fluorescent, whereas the hydrogenated product **12** did not fluoresce. Therefore, such a long conjugated system comprised of the two aromatic rings connected with the triple bond is necessary for fluorescence. Fluorescence properties of imidazo[4,5-*b*]pyridines **10** and **11** were compared with those of the purines **4** and **5** in aqueous acidic, neutral and alkaline solutions. Compound **11** afforded simple fluorescence excitation and fluorescence emission spectra under the different conditions (Figure 2). Excitation maxima of **11** appeared in a narrow range of 310−325 nm, not far from the excitation maxima of

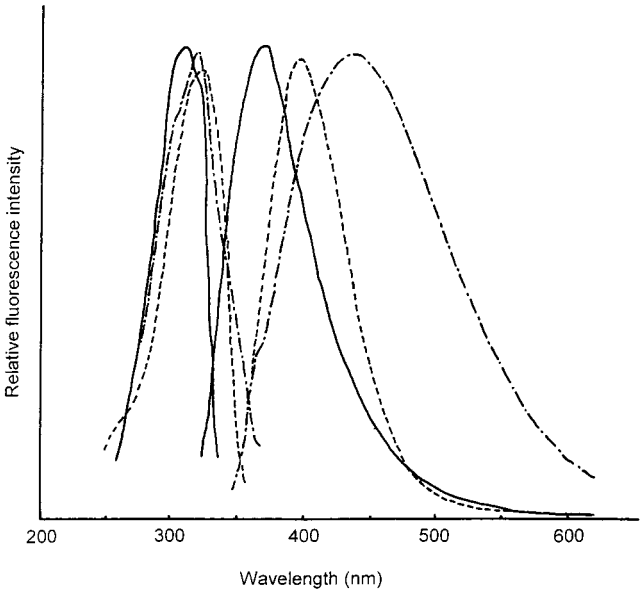


Figure 2. Fluorescence excitation and emission spectra of **11** in aqueous neutral (- - -), 0.1 N HCl (—) and 0.1 N NaOH (— - —) solutions. The relative fluorescence intensity is arbitrary for each solution.

Table 1. Fluorescent Properties of the Alkynyl Derivatives of Purines 4 and 5 and Imidazo[4,5-*b*]pyridines 10 and 11[a]

	H_2O		0.1 N HCl		0.1 N NaOH	
compd	ex (nm)	em (nm)	ex (nm)	em (nm)	ex (nm)	em (nm)
4	328	416 (7.4)	329	430 (2.3)	328	423 (3.9)
5	330	397 (10.2)	332	447 (1.5)	327	499 (715)
10	310	373 (15.6)	322	406 (5.8)	322	390 (14.3)
11	309	363 (14.8)	319	425 (3.6)	324	395 (375)

[a] Numbers in parentheses indicate the relative fluorescence intensities. Each compound (5×10^{-6} M) was excited at its maximum at 25 °C. The fluorescence intensity of each control was less than 0.04.

tyrosine and tryptophan. The riboside **10** and its free base **11** provided emission maxima at wavelength approximately 35−45 nm shorter than those of **4** and **5**, when excited at the excitation maximum of each compound in water (Table 1). In 0.1 N HCl solution, emission maxima of the four compounds shifted to longer wavelength compared to those in water with the decrease in fluorescence intensity. The most striking was the change in fluorescence intensity in 0.1 N NaOH solution. The free base **11** strengthened the intensity 25-fold compared to its intensity measured in water. Similarly, the purine base **5** gave more significant enhancement (70-fold). In contrast, the ribosides **4** and **10** gave slightly lower intensities than those measured in water. Thus, deprotonation of the imidazo[4,5-*b*]-pyridine or the purine ring under alkaline conditions is critical for the enhancement.

Biological Activities. Cytokinin activities of the imidazo[4,5-*b*]pyridines **10** and **11**, and the purines **4** and **5** were compared in three different bioassays. In a betacyanin bioassay, compounds **10** and **11** were stronger than **4** and **5**, respectively (Figure 3). The order of the activity was **11** > **10**, **5** > **4**. The imidazo[4,5-*b*]-pyridine base **11** exhibited a strong activity, its equiactive concentration (0.26 μM) being close to that of BA (0.1 μM), a standard compound in this bioassay.

The free base **11** was more active than kinetin, and slightly less active than BA in a tobacco callus bioassay

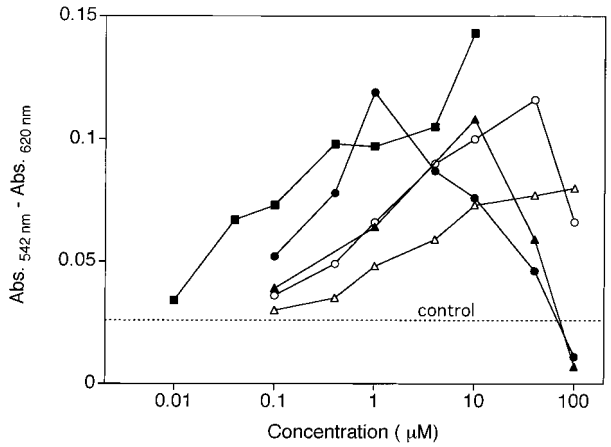


Figure 3. Effects of the alkynyl derivatives **4**, **5**, **10**, and **11** on betacyanin biosynthesis of detached *Amarathus* seedlings: BA (■); **4** (△); **5** (▲); **10** (○); **11** (●).

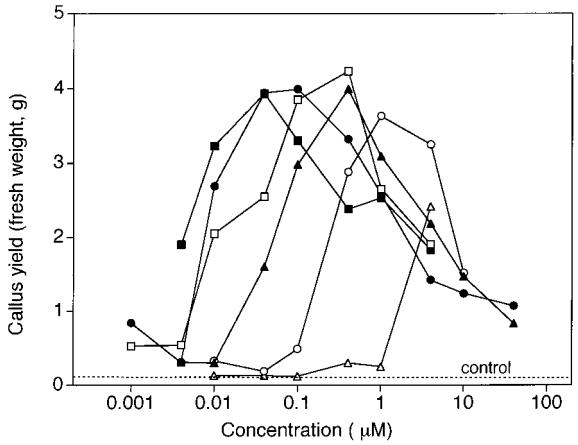


Figure 4. Effects of the alkynyl derivatives **4**, **5**, **10**, and **11** on tobacco callus growth: BA (■); kinetin; (□), **4** (△); **5** (▲); **10** (○); **11** (●).

(Figure 4). The maximum callus yield of **11** was almost the same as that of BA at the optimal concentration. On the other hand, its riboside **10** was less active than kinetin, but gave a maximum callus yield similar to that of BA. The imidazo[4,5-*b*]pyridine base **11** induced shoot formation in a high frequency at 40 μM, whereas its ribose **10** promoted it in a low frequency at the same concentration. The purines **4** and **5** were less active than **10** and **11**, respectively. The former were toxic at 40 μM. Cytokinin activities of BA, kinetin, **4**, **5**, **10**, and **11** were 0.0063, 0.021, 4.3, 0.32, 0.061, and 0.0095 μM, respectively. Thus, the order of the activity of the alkynyl derivatives was **11** > **10** > **5** > **4** in the bioassay. A minute structural change caused striking effects. The displacement of the nitrogen atom at the 1-position of **5** with a methine group resulted in about 35-fold enhancement in the activity, in accordance with the results of N^6-benzoyl-1-dezapurine (Matsubara et al., 1978). More significantly, similar displacement of the riboside **4** led to approximately a 70-fold increase in the activity. Since most alkynyl derivatives of purine, pyrimidine, and pyridine were weak or inactive, it was surprising to us that the alkynyl derivative **11** was almost as active as BA.

In a lettuce seed germination bioassay, however, **11** showed considerably lower activity compared to BA (Figure 5). The activities of the four alkynyl derivatives and BA in the lettuce seed germination bioassay were not parallel to their activities in the tobacco callus

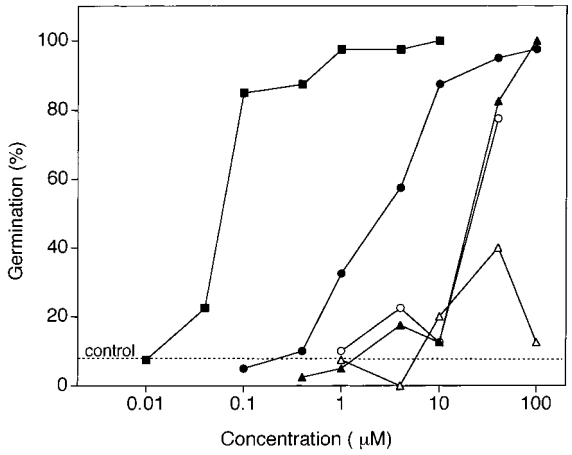


Figure 5. Effects of the alkynyl derivatives **4**, **5**, **10**, and **11** on lettuce seed germination: BA (■); **4** (△); **5** (▲); **10** (○); **11** (●).

bioassay, although there was a good linear relationship between the activities in the tobacco callus and the betacyanin bioassays, when they were plotted against each other (data not shown). The order of the activity, **11** > **5**, **10** > **4**, in the lettuce seed germination bioassay was the same as that in the betacyanin bioassay.

The finding of a highly active imidazo[4,5-*b*]pyridine derivative, **11**, clearly showed that high cytokinin activity is maintained, even though the two N^1- and N^6-atoms are replaced with carbon atoms. This suggests that the exocyclic nitrogen atom does not function as a hydrogen bond acceptor or a hydrogen donor, but orients the side chain in a direction favorable for the activity (Nishikawa et al., 1986). In addition, the nitrogen atom at the 1-position of purine may not play an essential role in the receptor binding because it is replaceable with a carbon atom, as shown in the previous and the present studies. Otherwise, the nitrogen−carbon atom change in purine ring may alter the basicity of the remaining nitrogen atoms to intensify the activity.

In this study, we synthesized a new cytokinin analogue, **11**, possessing both potent activity and strong fluorescence. It will find use in the studies of membrane transport and localization of cytokinins in plant cells or tissues.

MATERIALS AND METHODS

^{1}H NMR spectra were measured with a Hitachi R-90H (90 MHz) or a JEOL JNM-A500 (500 MHz) instrument at the Mie University Cooperative Research Center, chemical shifts being given in parts per million downfield from tetramethylsilane. UV spectra were recorded on a Shimazu UV 300 spectrometer, and IR spectra were taken with a Shimazu IR 470 spectrometer. LC−MS analyses were performed with a Thermoquest LCQ spectrometer by a APCI mode using methanol as solvent. Microanalyses were carried out with a Yanagimoto MT-3 CHN apparatus. Precoated silica gel plates 60 F_{254} and reversed-phase plates RP-8 from Merck were used for TLC, and Wakogel C-200 or Fuji Silisia BW-200 was used for column chromatography. Fluorescence spectra were measured with a Hitachi fluorospectrometer 650-60, and excitation and emission spectra were uncorrected.

7-Amino-3-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo-[4,5-*b*]pyridine was prepared as a syrup from 2,3-diaminopyridine by ribosylation of 7-nitroimidazo[4,5-*b*]pyridine (Delvin et al., 1995; Wenzel and Seela, 1996), followed by palladium reduction (Antonini et al., 1984; Jain et al., 1966; Cristalli et al., 1987). Both 6-phenylethynylpurine and its riboside were synthesized, as reported previously (Koyama et al., 1982).

Synthesis of Compounds. *7-Iodo-3-(2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (**8**). Method A.* To a mixture of **7** (500 mg, 1.27 mmol) in isoamyl nitrite (6.5 mL, 48.4 mmol) was added CH_2I_2 (6.0 mL, 74.5 mmol). The mixture was degassed and stirred under nitrogen with illumination with an unfrosted 200 W tungsten lamp at 90 °C for 2 h. It was diluted with CH_2Cl_2, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc−benzene, 1:2) to give the iodide **8** as a yellow syrup (485 mg, 76%): IR (KBr) 2930 (C−H), 1740 (C=O), 1554, 1486 (C=N, C=C) 1236, 1044 (C−O−C) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.08, 2.12, and 2.15 (9H, s, Ac), 4.4−4.5 (3H, m, 4′-H, 5′-H), 5.70 (1H, m, 3′-H), 6.03 (1H, m, 2′-H), 6.24 (1H, d, 1′-H, $J_{1',2'}$ = 5.5 Hz), 7.73 (1H, d, 6-H, $J_{5,6}$ = 5.2), 8.04 (1H, d, 5-H, $J_{5,6}$ = 5.2), 8.29 (1H, s, 2-H); LC−MS *m/z* (rel intens) 503.9 ([M + H]$^+$, 100), $C_{17}H_{18}IN_3O_7$ requires 503.2.

Method B. To a mixture of **7** (1.48 g, 3.77 mmol) in isoamyl nitrite (15 mL, 112 mmol) was added CH_2I_2 (12 mL, 149 mmol). The mixture was reacted under the conditions described in method A, except for illumination (Matsuda et al., 1992). Similar workup and chromatographic separation on silica gel as in method A gave the iodide (882 mg, 47%).

*7-Phenylethynyl-3-(2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (**9**).* To a mixture of **8** (471 mg, 0.936 mmol), $(PPh_3)_2PdCl_2$ (15 mg, 0.02 mmol), and CuI (8 mg, 0.04 mmol) in MeCN (10 mL) were added phenylacetylene (0.20 mL, 1.8 mmol) and triethylamine (0.10 mL, 0.72 mmol). The mixture was degassed, and stirred at 90 °C under nitrogen for 2 h. It was diluted with CH_2Cl_2, filtered, and concentrated in vacuo. Purification of the residue by silica gel column chromatography (EtOAc−benzene, 1:2) gave the alkynyl compound **8** as a pale yellow syrup (417 mg, 93%): IR (KBr) 2923 (C−H), 2200 (C≡C), 1740 (C=O), 1588, 1480 (C=N, C=C), 1223 and 1044 (C−O−C), 756 and 690 (Ph C−H) cm^{-1}; ^{1}H NMR($CDCl_3$) δ 2.09, 2.13, and 2.16 (9H, s, Ac), 4.37−4.40 (1H, m, 4′-H), 4.46−4.49 (2H, m, 5′-H), 5.72 (1H, m, 3′-H), 6.04 (1H, m, 2′-H), 6.30 (1H, d, 1′-H, $J_{1',2'}$ = 5.5 Hz), 7.37−7.42 (3H, m, $H_{m,p}$), 7.41 (1H, d, 6-H), 7.67−7.68 (2H, m, H_o), 8.28 (1H, s, 2-H), 8.39 (1H, d, 5-H, $J_{5,6}$ = 4.9 Hz); LC−MS *m/z* (rel intens) 478.1 (100, [M + H]$^+$), 220.3 (15), $C_{25}H_{23}N_3O_7$ requires 477.5.

*7-Phenylethynyl-3-(β-D-ribofuranosyl)-3H-imidazo[4,5-b]-pyridine(**10**).* A solution of **9** (501 mg, 1.05 mmol) in MeOH (50 mL) was saturated at 0 °C with ammonia and kept at 4 °C for 16 h. It was concentrated in vacuo to give the residue which was purified by silica gel column chromatography (EtOAc−MeOH, 20:1). Crystallization from EtOAc afforded the riboside **10** (327 mg, 89%) as colorless crystals: mp 95−98 °C; IR (KBr) 3380 (OH), 2920 (C−H), 2200 (C≡C), 1592 and 1488 (C=N, C=C), 1195 and 1076 (C−O−C) 756 and 688 (Ph C−H) cm^{-1}; UV λ_{max} (H_2O) 321^{sh} (ϵ 20 200), 302 (ϵ 27 400), λ_{min} 253 (ϵ 5850) nm; λ_{max} (0.1 N HCl) 320^{sh} (ϵ 22 500), 301 (ϵ 23 700), λ_{min} 255 (ϵ 4920) nm; λ_{max} (0.1 N NaOH) 322^{sh} (ϵ 21 600), 303 (ϵ 27 100), λ_{min} 254 (ϵ 5 230) nm; ^{1}H NMR (CD_3OD) δ 3.8−3.9 (2H, m, 5′-H), 4.21 (1H, s, 4′-H), 4.38 (1H, t, 3′-H), 4.83 (1H, t, 2′-H), 6.14 (1H, d, 1′-H, $J_{1',2'}$ = 4.9), 7.4 (4H, m, 6-H $H_{m,p}$), 7.7 (2H, m, H_o), 8.34 (1H, d, 5-H, $J_{5,6}$ = 4.9 Hz), 8.69 (1H, s, 2-H); LC−MS *m/z* (rel intens) 352.1 ([M + H]$^+$), 296.3 (39), 280 (20), 220 (46), $C_{19}H_{17}N_3O_4$ requires 351.4. Anal. Calcd for $C_{19}H_{17}N_3O_4 \cdot 0.5H_2O$: C, 63.33; H, 5.03; N, 11.66. Found: C, 63.14; H, 5.42; N, 9.99.

*7-Phenylethynylimidazo[4,5-b]pyridine (**11**).* The riboside **10** (100 mg, 0.285 mmol) was dissolved in a mixture of 0.2 N HCl (5 mL) and dioxane (5 mL) and heated at reflux for 2 h. The mixture was neutralized with saturated aqueous $NaHCO_3$, concentrated in vacuo, and purified by column chromatography (EtOAc−MeOH, 20:1) to give **11** as a pale yellow syrup (46.5 mg, 74%). Recrystallization from EtOAc yielded a colorless crystalline powder: mp 193−194 °C; IR (KBr) 2200 (C≡C), 1593 (C=N, C=C), 755 and 690 (Ph C−H) cm^{-1}; UV λ_{max} (H_2O) 322^{sh} (ϵ 20 700), 301 (ϵ 28 300), λ_{min} 252 (ϵ 5230) nm; λ_{max} (0.1 N HCl) 344^{sh} (ϵ 12 700), 318 (ϵ 20 500), λ_{min} 256 (ϵ 3850) nm; λ_{max} (0.1 N NaOH) 315 (ϵ 19 400), λ_{min} 275 (ϵ 7970) nm; ^{1}H NMR (CD_3OD) δ 7.44 (4H, m, 5-H, $H_{m,p}$) 7.71 (2H, m, H_o), 8.39 (1H, d, 6-H, $J_{5,6}$ = 4.9 Hz), 8.45 (1H, s, 2-H); LC−MS *m/z* (rel intens) 220.2 (M + H, 100), $C_{14}H_9N_3$ requires 219.24. Anal.

Calcd for $C_{14}H_9N_3$0.8 H_2O: C, 71.97; H, 4.57; N, 17.98. Found: C, 71.44; H, 4.29; N, 17.26.

*7-Phenylethyl-(3-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (**12**).* To a solution of the riboside **10** (30.0 mg, 0.0854 mmol) in EtOH (10 mL) was added 10% Pd/C (15 mg), and the mixture was stirred at room temperature under hydrogen for 24 h. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue (23.9 mg, 79%) was recrystallized from EtOAc to provide the phenylethyl derivative **12** as colorless crystals: mp 171−173 °C; IR (KBr) 3380 (OH) 2915 (C−H), 1204, 1088 (C−O−C), 766 and 716 (Ph C−H), cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.08 (2H, m, PhC−H), 3.33 (2H, m, PuC−H), 3.90 and 3.76 (2H, m, 5′-H), 4.20 (1H, m, 4′-H), 4.35 (1H, t, 3′-H), 4.84 (1H, t, 2′-H), 6.08 (1H, d, 1′-H, $J_{1,2}$ = 6.1 Hz), 7.1−7.2 (6H, m, Ph−H, 6-H), 8.21 (1H, d, 5-H , $J_{5,6}$ = 4.9 Hz), 8.58 (1H, s, 2-H). Anal. Calcd for $C_{19}H_{21}N_3O_4$: C, 64.21; H, 5.96; N, 11.81. Found: C, 63.47; H, 5.96; N, 11.67.

Betacyanin Bioassay. This bioassay was carried out in two replications by using seedlings of *Amaranthus caudatus* L., as reported previously (Nishikawa et al., 1986b). Briefly, 10 detached seedlings were incubated in a phosphate buffer (pH 6.3) containing varying amounts of test sample and L-tyrosine at 28 °C for 24 h in the dark, and the amount of betacyanin was measured as the differential absorbance between 542 and 620 nm. Cytokinin acivity was determined in terms of $C_{0.1\,\mu M\,BA}$ (μM). The averaged cytokinin activity was calculated from the data in two experiments. The standard error of the differential absorbance was 0.013.

Tobacco Callus Bioassay. Callus tissues derived from *Nicotiana tabacum* cv. Wisconsin No. 38 were grown on Linsmaier and Skoog medium at 28 °C for 40 days in the dark, as reported previously (Nishikawa et al., 1986b). Fresh weights of the callus tissues obtained in four replications were averaged, and cytokinin activity was determined as a defined concentration, $C_{1/2,max,K}$ (μM). The averaged cytokinin activity was calculated from the data in two experiments. The standard error of the fresh weight was 0.20 g.

Lettuce Seed Germination Bioassay. According to the reported method (Nishikawa et al., 1986b), seeds of *Lactuca sativa* cv. Great Lakes 366 were allowed to germinate on a filter paper moistened with water solution containing sample at 30.5 °C for 4 days in the dark. The germination of the control was almost inhibited at the temperature due to thermodormancy. The standard errors of the control and BA (0.1−10 μM) were 7.2% and 5.0%, respectively.

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