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FLUORESCENT CYTOKININS: STRETCHED-OUT ANALOGS OF N^6-BENZYLADENINE AND N^6-(Δ^2-ISOPENTENYL) ADENINE

MARK A. SPRECKER, ALAN G. MORRICE, BRUCE A. GRUBER and NELSON J. LEONARD
Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL 61801
and
RUTH Y. SCHMITZ and FOLKE SKOOG
Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, WI 53706, U.S.A.

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Key Word Index—Cytokinins; benzoadenines; *N*-(Δ^2-isopentenyl)-, *N*-benzyl-imidazoquinazolines; biological activity; fluorescence.

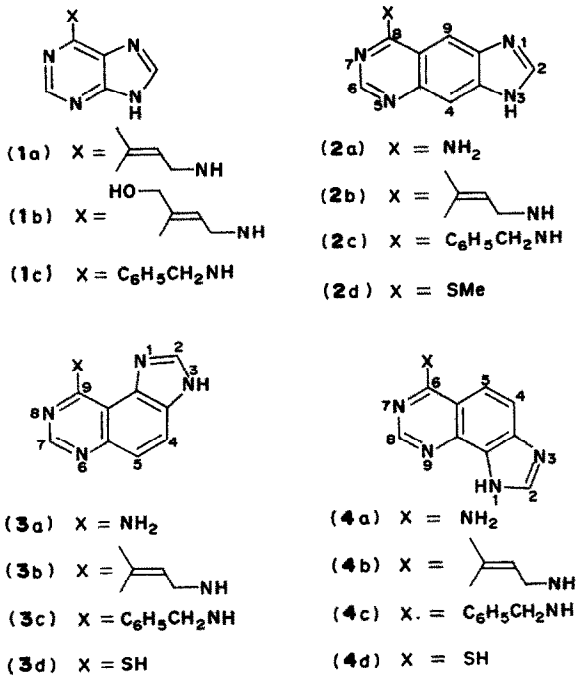
Abstract—We have synthesized and compared the cytokinin activities in the tobacco bioassay of a series of benzologs of 6-(3-methyl-2-butenylamino)purine (N^6-(Δ^2-isopentenyl)adenine) (**1a**) and 6-benzylaminopurine (N^6-benzyladenine) (**1c**). The linear benzo analogs 8-(3-methyl-2-butenylamino)imidazo[4,5-*g*]quinazoline (**2b**) and 8-benzylaminoimidazo[4,5-*g*]quinazoline (**2c**) are active, while 9-(3-methyl-2-butenylamino)imidazo[4,5-*f*]quinazoline (**3b**) and 6-(3-methyl-2-butenylamino)imidazo[4,5-*h*]quinazoline (**4b**) are slightly active and 9-benzylaminoimidazo[4,5-*f*]-quinazoline (**3c**) and 6-benzylaminoimidazo[4,5-*h*]quinazoline (**4c**) are inactive. Compounds **2b** and **2c** represent the first examples of active cytokinins containing a tri-heterocyclic moiety. The above series of compounds demonstrates structural factors that affect cytokinin activity. These compounds also have interesting fluorescence properties which could render them useful as probes to study the mechanism of cytokinin action.

INTRODUCTION

The systematic investigation of structure–activity relationships for cytokinins has led to the discovery of new compounds which, as promotors or antagonists of plant cell growth and differentiation, are potentially useful in discerning the nature of cytokinin activity [1,2]. For cytokinins that are adenine derivatives, most attention has been placed on the modification of the N^6 sidechain to obtain compounds related to naturally occurring cytokinins, i.e. 6-(3-methyl-2-butenylamino)purine (N^6(Δ^2-isopentenyl)adenine or i^{6}Ade) (**1a**) and 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (zeatin) (**1b**) [3–19], and to one of the first of the synthetic cytokinins, 6-benzylaminopurine (N^6-benzyladenine or bzl^{6}Ade) (**1c**). The effects of substituents placed at positions other than N^6 [4,7,20–22], as well as the modification of the central purine ring system by carbon–nitrogen interchange [23–26], also have been investigated. In general, these experiments have shown that the effects of progressive modification are additive and tend to decrease cytokinin activity [1]. Little work has been done in significantly altering the geometry of the central ring system, although it has been shown that substitution of quinoxaline or quinoline for purine can lead to moderate cytokinin activity [25].

Recently, we described the preparation of 8-aminoimidazo[4,5-*g*]quinazoline (**2a**), 9-aminoimidazo[4,5-*f*]-quinazoline (**3a**) and 6-aminoimidazo[4,5-*h*]quinazoline (**4a**), a family of structural analogs of adenine in which the pyrimidine ring and the imidazole ring of adenine have been "stretched-out" by the insertion of a benzene ring to form an extended purine model [26,27]. The three isomeric compounds, which we call *lin*-, *prox*-, and *dist*-benzoadenine, respectively, have hydrogen bonding sites similar to 1,N^6 in adenine, but have larger spatial requirements. (The prefix *lin.* refers to the linear disposition of the three rings in compound **2a**; *prox* for proximal and *dist* for distal refer to the relationship of the amino group in compounds **3a** and **4a** respectively, with respect to the imidazole ring.)

We were prompted to prepare cytokinin analogs based on the benzoadenines because of the biological activity demonstrated by compounds related to **2–4** when used as surrogates for natural purine substrates in enzymatic reactions [28]. Since there are no known cytokinins containing a central heterocyclic nucleus larger than bicyclic, the possibility of activity for the tricyclic benzoadenines would help define the limits of the spatial parameters for the central moiety in cytokinins. In addition, the demonstration of any differential cytokinin activity in the three systems could be relatable to the difference in relative positions of the pyrimidine and imidazole rings with respect to the central benzene ring. Finally, the substituted benzoadenines have fluorescence properties which could render them useful in binding studies and in probing the molecular function of cytokinins, which appear to be active at the small molecule level, i.e. without being incorporated into *t*RNA [29].



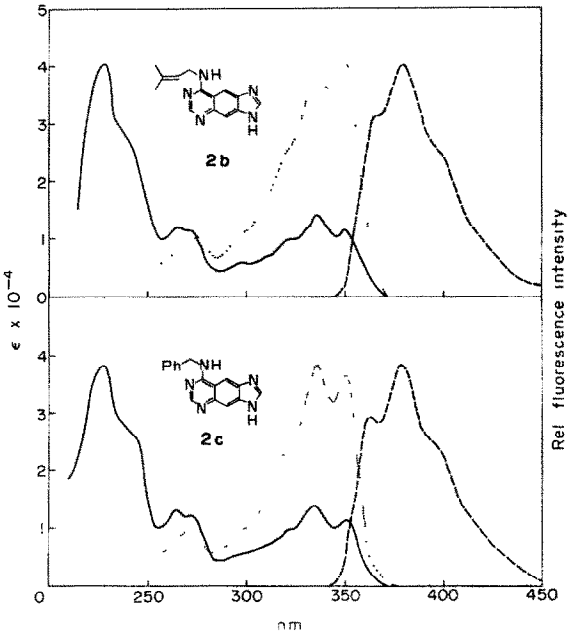


Fig. 1. The fluorescence and ultraviolet spectra of **2b** and **2c** in EtOH. (——) Ultraviolet absorption; (· · · · · ·) fluorescence excitation; (— — —) technical fluorescence emission.

RESULTS AND DISCUSSION

Synthesis

The benzyl derivatives **2c–4c** and the Δ^2-isopentenyl derivatives **2b–4b** were chosen as cytokinin targets. They were prepared by heating an ethanolic solution of the corresponding methylmercapto (**2d**) [27] or mercapto (**3d** and **4d**) [28] precursors with benzylamine or 3-methyl-2-butenylamine at 200°.

Fluorescence

The fluorescence spectra of the linear derivatives **2b** and **2c** are shown in Fig. 1. Both compounds (as well as the angular benzoadenines listed in Table 1) exhibit complex excitation and emission spectra. The excitation maxima are found at longer wavelength than the excitation bands for fluorescent protein constituents such as tryptophan and tyrosine. Thus, the benzoadenine derivatives can be excited selectively, and their fluorescence can be monitored in the presence of proteins or nucleic acids. The linear and proximal compounds **2** and **3** (**b**,**c**) have appreciable quantum yields (Table 1). The distal derivatives **4** (**b** and **c**), however, show very weak fluorescence (ϕ = 0·003 and 0·007, respectively), probably due to quenching *via* hydrogen transfer between N-1 and N-9. The quantum yields and fluorescence lifetimes of the linear compounds were determined in several solvents. These parameters generally increase with decreasing solvent polarity, implying that the fluorescence intensity of the benzoadenines would increase in hydrophobic environments. Thus, the fluorescence can be monitored for possible measurement of the binding of **2b** or **2c** to proteins, lipids, or etc. [30–32]. The fluorescence lifetimes, although relatively short, are longer in media of decreasing polarity. In a sufficiently hydrophobic region, such as an interior region of a protein or in a membrane, they may become sufficiently long to study the dynamics of the interaction of benzoadenine derivatives by fluorescence depolarization [33,34].

Table 1 Fluorescence data

Compound	Solvent	Excitation > 300, nm (uncorr.)*	Emission, nm (uncorr.)*	τ (nsec)†	Φ
2b	H_2O	320(*sh*), 335, 350	365(*sh*), 380, 395(*sh*)	0·9	0·070‡
	EtOH	320(*sh*), 335, 350	365(*sh*), 380, 395(*sh*)	1·7	0·290‡
	Dioxan	320(*sh*), 335, 350	365, 380, 395(*sh*)	2·0	0·330‡
2c	H_2O	320(*sh*), 335, 350	365(*sh*), 380, 395(*sh*)	1·3	0·140‡
	EtOH	320(*sh*), 335, 350	365(*sh*), 380, 395(*sh*)	1·8	0·260‡
	Dioxan	320(*sh*), 335, 350	365, 380, 395(*sh*)	2·2	0·290‡
3b	H_2O	325, 340	350, 362, 378(*sh*)	1·0	0·090‡
3c	H_2O	325, 338	350, 362, 378(*sh*)	2·0	0·120‡
4b	H_2O	302(*sh*), 312	330(*sh*), 340, 355(*sh*)	1·5	0·003¶
4c	H_2O	302(*sh*), 312	328(*sh*), 340, 355(*sh*)	1·7	0·007¶

* ± 2 nm.: † For fluorescence lifetime determinations, see experimental section. ‡ Absolute quantum yields measured relative to quinine in 1 N H_2SO_4 (Φ = 0·70). ¶ Absolute quantum yield measured relative to **3b**.

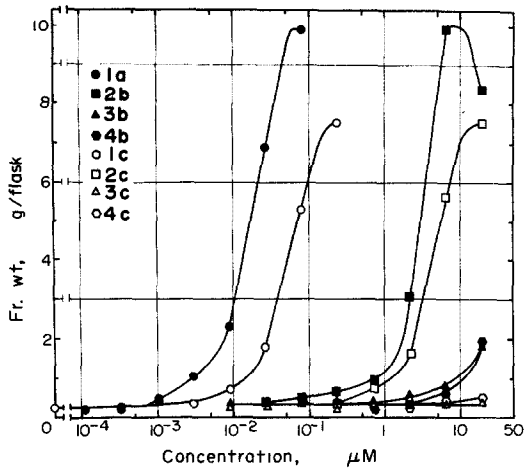


Fig. 2. Fresh weight yields of tobacco callus tissue cultured on six cytokinin analogs and on N^6-isopentenyladenine and N^6-benzyladenine. The compounds are numbered as in the text.

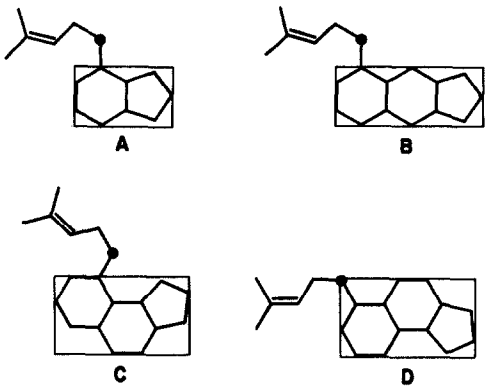


Fig. 4. Geometrical relationship of sidechains and heteronuclei.

Cytokinin activity

Cytokinin activity was determined by the tobacco bioassay [35,36], on the basis of fresh weight yields of callus obtained with serial concentrations of the test substances. The growth curves presented in Fig. 2 and the summary of relative activities in Fig. 3, show that the linear benzoadenines, **2b** and **2c**, possess cytokinin activity. The Δ^2-isopentenyl derivatives of the angular benzoadenines, **3b** and **4b**, exhibit very low activity, while the corresponding benzyl derivatives, **3c** and **4c**, show virtually no activity over the tested concentration range. Thus, we have demonstrated that a tricyclic central nucleus is not incompatible with cytokinin activity, especially when the three rings are arranged in linear array. The differential cytokinin activity observed for the linear and angular benzoadenine derivatives may be interpreted in several ways. If a cross section of the central aromatic portion of an adenine- or benzoadenine-derived cytokinin is enclosed in a rectangle (Fig. 4), the rectangle enclosing the *lin*-benzoadenine nucleus (**B**) is 1·7 times larger in area than the corresponding rectangle for adenine (**A**), while the rectangles enclosing the *prox*- and *dist*-benzoadenines (**C** and **D**) are 2·0 times as large as that for adenine. Thus, activity may be related to the area defined by a cross section of the central nucleus; i.e. adenine-derived cytokinins are more active than those derived from the larger *lin*-benzoadenines, which are in turn more active than those representing the still larger angular benzoadenine cytokinin analogs. Another way to relate the differential cytokinin activity observed for **2–4 (b,c)** to structural morphology is to examine the geometrical relationship of the sidechains and rectangularly enclosed heteronuclei in Fig. 4. The relative positions of these components in **A** and **B** (**1a** and **2b**) are mutually correspondent. By contrast, for the less active angular analogs **3b** and **4b** (represented by **C** and **D** in Fig. 4), the side-chains have differing spatial relationships with the central ring.

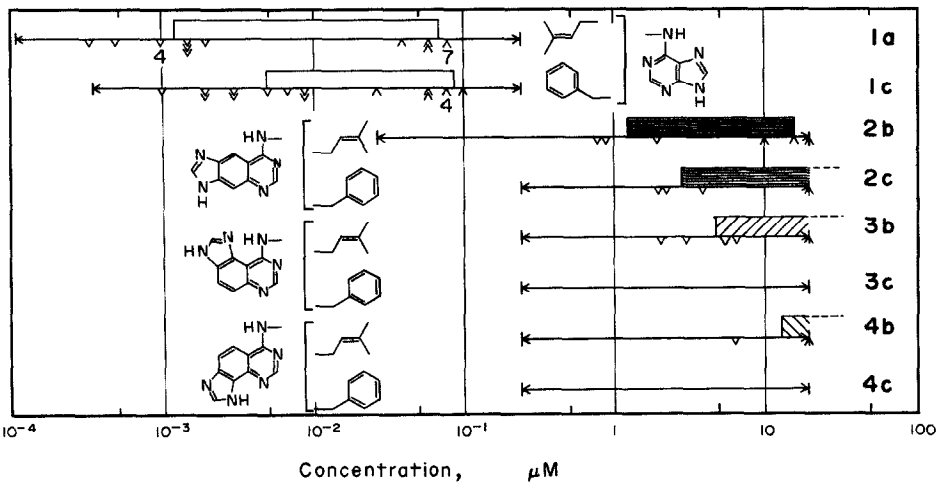


Fig. 3. Relative cytokinin activities of adenine derivatives and benzoadenine derivatives. The base lines represent the tested concentration ranges, and the arrows under the base lines represent the start and end points (in individual experiments) of the concentration range over which growth increases as a nearly linear function of the log of concentration of added cytokinin. Bars represent the average range of the linear growth response.

The substituted linear benzoadenines, **2b** and **2c**, are less active than their respective purine models, i^{6}Ade (**1a**) and bzl^{6}Ade (**1c**), as evidenced by the greater concentrations of **2b** or **2c** needed to obtain growth comparable to that for **1a** or **1c**. However, **2b** and **2c** permit the same maximum yields to be obtained as do their purine models. As in the substituted adenines (Figs. 2 and 3), the compounds containing benzylamino sidechains are less active than those containing Δ^2-isopentenylamino sidechains.

The *N*-isopentenyl-*prox*- and *dist*-benzoadenines, **3b** and **4b**, respectively, retain some activity, although they are less active than the corresponding linear compound **2b**. The angular *N*-benzylamino-*prox*- and *dist*-benzoadenines, **3c** and **4c**, were essentially inactive at the concentrations tested. These results tend to emphasize the importance of variations in individual sub-units as well as overall structure in the attainment of biological activity [21]. The difference in cytokinin activity due to changing the sidechain from Δ^2-isopentenylamino to benzylamino seems to operate quasi-independently of other structural changes in the molecule.

The synthesis of the substituted benzoadenines and the resultant determination of differential cytokinin activity provides a useful basis for determining size limitations and geometrical requirements for cytokinins. The fluorescence properties of the active linear benzoadenines **2b** and **2c** suggest that these compounds may be useful probes for detecting sites and modes of cytokinin action.

EXPERIMENTAL

Synthesis of test substances. All melting points are corrected. The synthesis of **2c** has been previously reported. The spectral data are listed for comparative purposes.

General synthetic procedure. A sealed tube containing ca 2–3 mmol of amine and 1 mmol of **2d**, **3d**, or **4d** in 8 ml EtOH was heated at 200° for 48 hr. The benzylamino derivatives **2b–4b** separated, upon cooling, as dark crystals, and were recrystallized from EtOH (charcoal). The isopentenyl derivatives **2c–4c** were obtained similarly. Compounds were recrystallized further to ensure purity at the level required for the determination of fluorescence lifetimes and yields.

8-*Benzylaminoimidazo*[4,5-g]*quinazoline* (**2c**). NMR (60 M Hz, DMSO-d_6): δ 4·83 (2H,*s*), 7·08–7·50 (5H,*m*), 7·86 (1H,*s*), 8·37 (1H,*s*), 8·48 (1H,*s*), 8·65 (1H,*s*); UV λ_{max} (95% EtOH) nm (ϵ): 227 (38200), 236 (*sh*), 264 (13000), 272 (12200), 322 (*sh*), 334 (14000), 351 (11300); λ_{max} (0·1 N HCl) (95% EtOH) nm (ϵ): 217 (34500), 237 (*sh*), 265 (7800), 324 (*sh*), 338 (19800), 354 (20900); λ_{max} (0·1 *N* NaOH) (95% EtOH) 244 nm (42200), 248 (*sh*), 270 (*sh*), 277 (31400), 324 (12500), 349 (9500), 365 (7900).

8-(3-*Methyl*-2-*butenylamino*)*imidazo*[4,5-g]*quinazoline* (**2b**). Yield, 70%; mp 287–289°; NMR (60 M Hz, DMSO-d_6): δ 1·75 (6H,*s*), 3·0–4·0 (1H,*m*), 4·0–4·3 (2H,*m*), 5·2–6·6 (1H,*m*), 7·86 (1H,*s*), 8·2–8·7 (1H,*m*), 8·46 (H,*s*), 8·55 (1H,*s*), 8·67 (1H,*s*); UV λ_{max} (95% EtOH) nm (ϵ): 228 (40300), 239 (*sh*), 245 (12000), 261 (*sh*), 297 (5800), 310 (*sh*), 324 (*sh*), 335 (14200), 350 (11600); λ_{max} (0·1 *N* HCl) (95% EtOH) nm (ϵ): 218 (38700), 237 (*sh*), 263 (9000), 288 (*sh*), 326 (*sh*), 338 (3900), 354 (4100); λ_{max} (0·1 *N* NaOH) (95% EtOH) nm (ϵ): 244 (43300), 249 (41000), 270 (25800), 278 (28700), 315 (9500), 326 (12500), 348 (9900), 365 (8300); MS (probe) 70 eV *m/e*; 253 (M$^+$). (Found: C, 66·27; H, 6·06; 27·63. $C_{14}H_{15}N_5$ requires: C, 66·38; H, 5·97; N, 27·65%).

9-*Benzylaminoimidazo*[4,5-f]*quinazoline* (**3c**). Yield, 89%; mp 270° (d); NMR (60 M Hz, TFA). δ 5·32 (1H,*s*), 7·25–7·55 (5H,*m*), 8·13 and 8·60 (2H, AB *q*, *J* 9 Hz), 8·93 (*s*), 9·45 (*s*); UV λ_{max} (pH 7) nm (ϵ): 238 (12300), 265 (17900), 277 (*sh*), 311 (*sh*), 323 (9500), 336 (8600); λ_{max} (pH 1) nm (ϵ): 232 (13100), 276 (20400), 307 (*sh*), 312 (*sh*), 323 (10500), 329 (*sh*); λ_{max} (pH 13) 272 (27200), 296 (*sh*), 332 (8500), 343 (*sh*); MS (probe) 70 eV *m/e*: 275 (M$^+$). Found: C,69·92; H, 4·80; N, 25·42. $C_{16}H_{13}N_5$ requires: C, 69·80; H, 4·76; N, 25·44%).

9-(3-*Methyl*-2-*butenylamino*)*imidazo*[4,5-f]*quinazoline* (**3b**). Yield, 88%; mp 217–220° (d); NMR (60 M Hz, DMSO-d_6): δ 1·78 (6H,*s*), 4·12–4·47 (2H,*m*), 5·29–5·68 (1H,*m*), 7·58 and 8·02 (2H, AB*q*, *J* 9 *Hz*), 8·53 (1*H*,*s*), 8·56 (1H,*s*), 8·75–9·05 (1H,*m*): UV λ_{max} (pH 7) nm (ϵ): 230 (*sh*), 238 (12800), 262 (13200), 311 (*sh*), 322 (9600), 337 (*sh*); λ_{max} (pH 1) nm (ϵ): 231 (13400), 276 (17300), 309 (*sh*), 321 (*sh*), 332 (*sh*); λ_{max} (pH 13) nm (ϵ): 273 (26000), 297 (*sh*), 331 (8600), 341 (*sh*); MS (probe) 70 eV *m/e*: 253 (M$^+$). (Found: C, 66·38; H, 5·97; N, 27·65. $C_{14}H_{15}N_5$ requires: C, 66·38; H, 5·97; N, 27·65%).

6-*Benzylaminoimidazo*[4,5-h]*quinazoline* (**4c**). Yield, 88%; mp 290–291°; NMR (100 M Hz, DMSO-d_6): δ 4·82 (2H, *d*, *J* 6 Hz), 7·18–7·48 (5H,*m*), 7·79 and 8·09 (2H, AB*q*, *J* 9 Hz), 8·37 (1H,*s*), 8·58 (1H,*s*), 8·77 (1H,*t*, *J* 6 Hz); UV λ_{max} (95% EtOH) nm (ϵ): 228 (26600), 253 (41800), 300 (14100), 314 (12600); λ_{max} (0·1 *N* HCl) (95% EtOH) nm (ϵ): 222 (20200), 261 (33500), 314 (14700); λ_{max} (0·1 *N* NaOH) (95% EtOH) nm (ϵ): 266 (64200), 314 (11300); MS (probe) 70 eV *m/e*: 285 (M$^+$). (Found: C, 69·99; H, 4·68; N, 25·29, $C_{16}H_{13}N_5$ requires: C, 69·80; H, 4·76; N, 25·44%).

6-(3-*Methyl*-2-*butenylamino*)*imidazo*[4,5-h]*quinazoline* (**4b**). Yield, 70%; mp 253–256° (d); NMR (100 M Hz, DMSO-d_6): δ 1·72 (3H,*s*), 1·75 (3H,*s*), 4·17 (2H, *t*, *J* 6 Hz), 5·38 (1H,*m*), 7·73 and 8·01 (2H, AB*q* *J* 9 Hz), 8·23 (1H,*m*), 8·32 (1H,*s*), 8·57 (1H,*s*); UV λ_{max} (95% EtOH) nm (ϵ): 227 (28200), 253 (36200), 301 (14900), 314 (13600), 316 (*sh*); λ_{max} (0·1 *N* HCl) (95% EtOH) nm (ϵ): 222 (21000), 233 (*sh*), 261 (34400), 315 (15100), 328 (*sh*); λ_{max} (0·1 *N* NaOH) (95% EtOH) nm (ϵ): 266 (83100), 312 (12300); MS (probe) 70 eV *m/e*: 253 (M$^+$). (Found: C, 66·44; H, 5·99; N, 27·17. $C_{14}H_{15}N_5$ requires: C, 66·38; H, 5·97; N, 27·65%).

Fluorescence. Technical fluorescence emission and fluorescence excitation spectra were measured on a Hitachi Perkin–Elmer MPF-2-A spectrofluorometer. The technical fluorescence spectra obtained at 25° were not corrected for monochrometer efficiency and photomultiplier response. Fluorescence lifetimes were determined at 25° using the cross-correlation spectrofluorometer described by Spencer and Weber [37,38]. The exciting light was modulated at 14·2 MHz and was filtered through a monochrometer and a CS-7-54 Corning filter. The emission was observed through a CS-O-52 Corning filter. Fluorescence lifetime determinations by both phase and modulation were identical to within 0·3 nsec for compounds with lifetimes greater than 1 nsec. Below 1 nsec, only phase determinations are reliable. The relative quantum yield of each compound was calculated from the observed absorbance (at 338 nm for **2b**, **2c**, **3b**, and **3c** and 310 nm for **4b** and **4c**) and the area of the emission spectra. Quinine sulfate in 1·0 N H_2SO_4 was used as a reference compound for **2b**, **2c**, **3b**, and **3c**. The quantum yield of the reference is 0·70 [39]. The quantum yields of **4b** and **4c** were determined relative to **3b**.

Bioassay procedures. Cytokinin activities were determined in the tobacco bioassay and are based on the fr. wt yields of cytokinin-dependent callus tissue. Synthetic compounds were tested on tissue grown on 50 ml of medium as described [36]. To avoid degradation by heat, the compounds were dissolved in Me_2SO and added to the autoclaved nutrient agar medium just before it solidified [40].

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