Antisenescence Activity of 4,5-Disubstituted Imidazoles: New Cytokinin Mimics

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A series of 4,5-disubstituted imidazoles was synthesized for evaluation as potential antisenescence agents. Both imidazolecarboxamides and imidazolylbenzamides show significant chlorophyll retention activity in an excised wheat leaf bioassay. Structure–activity relationships have been derived for both compound types. In the imidazolecarboxamide series, replacement of the 4(5)-cyano group by a methyl group significantly enhanced antisenescence activity. In contrast, a 4(5)-cyano group afforded optimum activity in the imidazolylbenzamide series. Substitution by other electron-withdrawing groups such as nitro or carboxamido at the 4(5)-position afforded less active compounds. The most active compound in this study, *N*-(5-cyano-1*H*-imidazolyl)benzamide (**14**), was more active than kinetin in the wheat leaf chlorophyll retention bioassay and may represent a new structural type of cytokinin mimic.

Cytokinins are known to retard the process of senescence, including protein, nucleic acid, and chlorophyll degradation in excised leaf tissue (Sabater, 1985). Historically, however, structure–activity relationships of natural and synthetic cytokinins have been based on cell division bioassays such as tobacco or soybean callus growth stimulation. (Okamoto et al., 1974; Iwamura et al., 1985; Kurosaki et al., 1981; Mok et al., 1982; Takahashi et al., 1978).

Cytokinin activity has been reported for at least four different classes of compounds: heterocyclic ureas and amides, pyrimidines, and modified purine compounds (Matsubara, 1980). Representatives of all of these classes of cytokinin mimics also show chlorophyll retention activity in excised wheat leaf tissue. (Green, C. M., unpublished results). Under normal conditions, chlorophyll retention can be used as a indicator of tissue senesence. We have used a wheat leaf chlorophyll retention bioassay (Kuhnle et al., 1977; Henrie et al., 1988) to discover and optimize antisenescence activity of two series of 4,5-disubstituted imidazoles.

MATERIALS AND METHODS

Synthesis of Chemicals. Methods used to prepare the target imidazole derivatives are outlined in Scheme I (Cavender, 1986). Amides were prepared by one of two methods described below. Novel compounds were characterized by NMR, IR, and combustion analyses. Melting points are uncorrected.

Method A. 5-Methyl-N-phenyl-1H-imidazole-4-carboxamide (*11*) (mp 230 °C dec) was prepared from aniline and 5-methyl-4-imidazolecarbonyl chloride by the method of Godefroi et al. (1964); mp 259–260 °C. Anal. Calcd for $C_{11}H_{11}N_3O$: C, 65.65; H, 5.51; N, 20.88. Found: C, 64.98; H, 5.33; N, 20.77. Compounds **9** and **10** were prepared by this procedure.

Method B. N-(5-Cyano-1H-imidazol-4-yl)benzamide (*14*). Benzoyl chloride (1.1 g, 0.0078 mol) was added dropwise to a stirred solution of 0.81 g (0.0075 mol) of 4-amino-1*H*-imidazole-5-carbonitrile (**23**) in 15 mL of dry pyridine. After 1 h at reflux, pyridine was removed at reduced pressure. The residue was treated with 25 mL of saturated aqueous sodium bicarbonate solution. The residual solid was washed with water and recrystallized from methanol/ethyl acetate to give 0.41 g (26%); mp 226–230 °C [lit. mp 223–225 °C (Okumura et al., 1973)]. Anal. Calcd for $C_{11}H_8N_4O$: C, 62.26; H, 3.80; N, 26.40. Found: C, 62.51; H, 3.97; N, 26.22. Compounds **15–20** were prepared in a similar manner.

5-[(Phenylmethyl)amino]-1H-imidazole-4-carbonitrile (*21*). A solution of 4-amino-1*H*-imidazole-5-carbonitrile (**23**; 1.1 g, 0.01 mol) and benzaldehyde (1.1 g, 0.01 mol) in 25 mL of methanol was stirred overnight at ambient temperature and then heated on a steam bath for 20 min. The cooled solution was treated with 25 mL of ether and the insoluble portion removed by filtration. Concentration of the filtrate and recrystallization from toluene/butanol afforded 0.92 g (47%) of 5-[(phenylmethyl)imino]-1*H*-imidazole-4-carbonitrile, mp 200–202 °C. Reduction of the imine (0.7 g 0.004 mol) with sodium cyanoborohydride (0.5 g, 0.008 mol) in methanol (10 mL) and 1 M acetate buffer (pH 6, 5 mL) afforded benzylamine **21** (0.24 g) in 30% yield after silica gel chromatography using diethyl ether as eluant; mp 95–97 °C. Anal. Calcd for $C_{11}H_{10}N_4$: C, 66.64; H, 5.09; N, 28.27. Found: C, 66.40, H, 5.13; N, 28.21.

N-Phenyl-N'-(5-cyano-1H-imidazol-4-yl)urea (*22*). A solution of phenyl isocyanate (1.3 g, 0.01 mol) and 4-amino-1*H*-imidazole-5-carbonitrile (**23**; 1.1 g, 0.01 mol) in 30 mL of 2-butanone was refluxed for 10 h. The solvent was removed, the residue suspended in ether, and the product removed by filtration. After being washed three times with ether, the solid was dissolved in ethanol/toluene and insoluble materials were removed by filtration. Concentration of the filtrate afforded 0.93 g (41%) of urea **22**, mp 163–166 °C dec. IR and NMR spectral data were consistent with the proposed structure.

Wheat Leaf Antisenescence Bioassay. The wheat leaf antisenescence bioassay reported by Kuhnle and coworkers (1977) was modified as described below. Wheat (*Triticum aestivum* var. Prodax) seeds are planted in a 50:50 mixture of vermiculite and sand, placed in a growth chamber (23 °C, 15-h days, 19 °C night, relative humidity 50%, 400 $\mu E \cdot M^{-2} \cdot s^{-1}$), and watered three times a day with tap water.

Stock solutions (10^{-4} M) of the test chemicals are prepared by dissolving the compound in 10 mL of the appropriate solvent (acetone, water, dimethyl sulfoxide) and adding 90 mL of distilled water. Test solutions are prepared by dilution of the stock solution with distilled water. The maximum solvent content in a test solution is 1% and does not affect the performance of the bioassay.

A control (three replicates) containing distilled water was run in each test. Kinetin was included as a standard in each test, usually at three concentrations in the range

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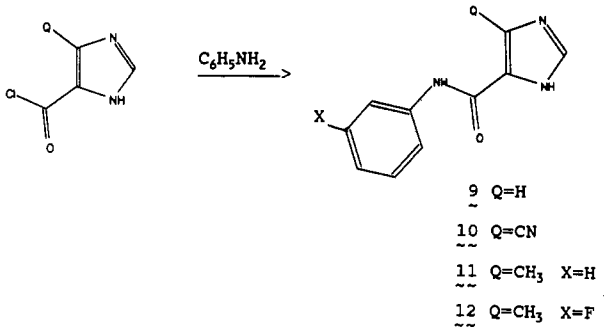
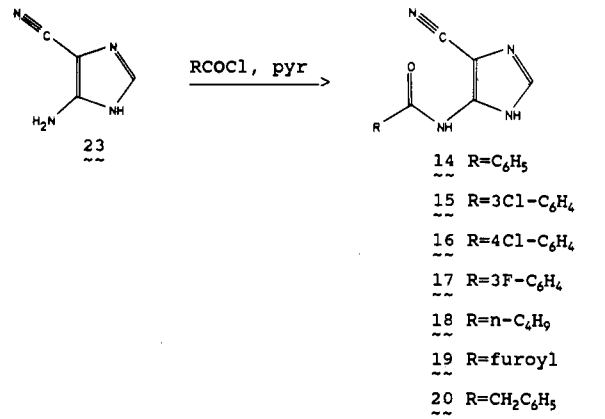
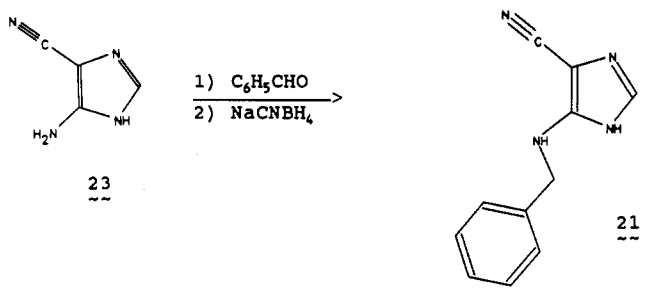
Scheme I. Synthesis of 4,5-Disubstituted Imidazoles

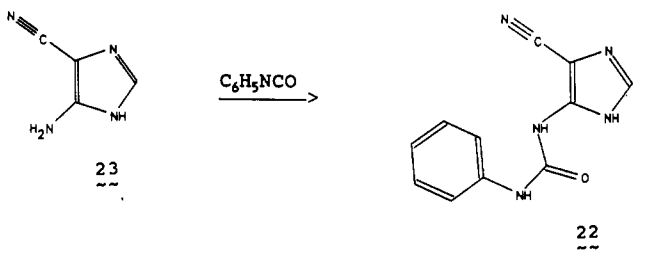
Method A



Method B







of 10^{-5}–10^{-8} M. The wheat leaf antisenescence bioassay consists of placing five weighed, 8-day-old primary wheat leaves, cut base down, in a vial containing 10–15 mL of an aqueous solution of the test chemical. All concentrations of compounds to be tested, including kinetin, are replicated three times. The vials containing the wheat leaves are incubated in the dark at 30 °C for 4 days. The zero time (ZT) treatments are placed in the freezer at 0 °C at the beginning of the incubation.

At the end of the incubation, each replicate, as well as the ZT treatments, are extracted with 10 mL of methanol at 65 °C for 10 min. The absorbance of each methanol extract at 652 nm is recorded. The data are converted to micrograms of chlorophyll/gram fresh weight by the following equation (MacKinney, 1941):

$$\frac{A_{652}}{\text{g fr wt}} \times 299 = \mu\text{g chlorophyll/g fr wt}$$

Table I. Antisenescence Activity of Selected Purine and Urea Cytokinins

no.	name	% ZT[a] (10^{-5} M)	pSI_{50}[b]
1	kinetin	75[c]	6.1[c]
2	*cis*-zeatin	45	4.9
3	dihydrozeatin riboside	62	5.5
4	kinetin riboside	72	6.3
5	*trans*-zeatin riboside	74	5.7
6	diphenylurea	26	
7	thidiazuron[d]	75	7.9
8	*N*-(2-chloropyridinyl)-*N'*-phenylurea	66	6.0

[a] Percent chlorophyll present at zero time, the beginning of the incubation. [b] –log [molar concentration] required to retain half (50%) of the chlorophyll initially present. [c] Average of 12 determinations. [d] *N*-Phenyl-*N'*-1,2,3-thiadiazol-5-ylurea.

A general linear models procedure using least squares was used to analyze these converted values. Test coefficients of variance (CV) averaged 15.4% ± 2.6% over 12 tests.

Chlorophyll retention is expressed as percent zero time (% ZT), obtained by dividing the mean of each test concentration in micrograms of chlorophyll/gram fresh weight by the respective experimental ZT mean in micrograms of chlorophyll fresh weight. The average percent ZT for the distilled water controls was 14.0% ± 7.5% (n = 12).

Initial comparisons of biological activity are based on chlorophyll retention (% ZT) at 10^{-5} M. The compounds are grouped into three categories:

% ZT	biol act.
>50	very active
30–50	active
<30	inactive

Of the compounds considered very active, additional dose–response information was generated to allow calculation of a pSI_{50} value, defined as –log [molar concentration] required to retain half (50%) of the chlorophyll initially present after a 4-day, 30 °C dark incubation. Kinetin, included in each test as a standard, had a pSI_{50} value of 6.1 ± 0.3 (n = 12).

RESULTS AND DISCUSSION

Purine Cytokinins. Eleven commercially available purine-based cytokinins were tested in the wheat leaf antisenescence bioassay to determine the response and the sensitivity of this system to structural variations. The pSI_{50} values of five of these eleven cytokinins range from 4.9 to 6.3 and represent an approximately 25-fold difference in activity (Table I).

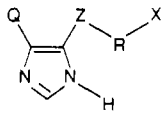
Three ureas cited as cytokinin mimics in the literature were also examined in the wheat leaf antisenescence bioassay (Table I). Diphenylurea (**6**) was not active at 10^{-5} M (28%) and did not warrant calculation of a pSI_{50} value. The pSI_{50} values of the other ureas are 6.0 and 7.9 and represent an 80-fold difference in activity. The wheat leaf antisenescence bioassay is sensitive to structural variation. In fact, it has been used to develop quantitative structure–activity relationships for the antisenescence activity of a series of pyridinylureas and their *N*-oxides (Henrie et al., 1988).

4,5-Disubstituted Imidazoles. We have discovered and optimized two series of 4,5-disubstituted imidazoles with enhanced activity using the wheat leaf antisenescence bioassay.

Imidazolecarboxamides. Compounds **9–11** are representative imidazolecarboxamides (Z = CONH, R = C_6H_4, X = H; Table II).

Carboxamide **9** (Q = H), reported by Okamoto et al. (1974) to stimulate the growth of tobacco callus at an

Table II. Structures and Antisenescence Activity of 4,5-Disubstituted Imidazoles



no.	Q	Z	R	X	% ZT[a] (10^{-5} M)	pSI_{50}[b]
9	H	CONH	C_6H_4	H	42	
10	CN	CONH	C_6H_4	H	35	
11	CH_3	CONH	C_6H_4	H	63	5.4
12	CH_3	CONH	C_6H_4	3-F	55	6.0
13	$CONH_2$	NHCO	C_6H_4	H	28	
14	CN	NHCO	C_6H_4	H	71[d]	7.1[d]
15	CN	NHCO	C_6H_4	3-Cl	71	6.4
16	CN	NHCO	C_6H_4	4-Cl	76	6.1
17	CN	NHCO	C_6H_4	3-F	78	7.0
18	CN	NHCO	n-C_4H_8	H	60	5.3
19	CN	NHCO	2-C_4H_2O[c]	H	54	5.1
20	CN	NHCO	$CH_2C_6H_4$	H	58	5.6
21	CN	$NHCH_2$	C_6H_4	H	40	
22	CN	NHCO	NHC_6H_4	H	29	

[a] Percent chlorophyll present at zero time, the beginning of the incubation. [b] -log [molar concentration] required to retain half (50%) of the chlorophyll initially present. [c] Furfuryl. [d] Average of five determinations.

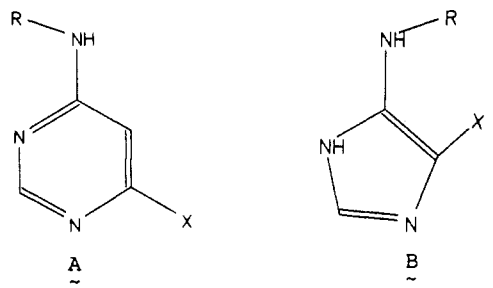


Figure 1. Relationship of pyrimidine- and imidazole-based cytokinin mimics.

optimum concentration of 10^{-4} M, has only moderate activity in the wheat leaf antisenescence bioassay at 10^{-5} M. Carboxamide **10**, where Q = H has been replaced by Q = CN, an electron-withdrawing group, is also active at 10^{-5} M. A significant increase in chlorophyll retention activity is observed for imidazole **11** where Q = CH_3, an electron-donating group, has been incorporated into the molecule. An additional 4-fold increase in activity is observed for substitution of fluorine for hydrogen in the meta position of the aromatic ring. The unique ability of meta fluorine substitution to increase cytokinin activity has been observed previously (Henrie et al., 1988).

Imidazolylbenzamides. Stimulation of tobacco callus growth by various 6-amino-substituted pyrimidine derivatives was reported by Takahashi et al. (1978). The cytokinin activity of pyrimidinylbenzamides **A** (Figure 1), as well as other derivatives, was enhanced by addition of an electron-withdrawing group at the pyrimidine 4-position. We have prepared a novel series of compounds **B** where the pyrimidine ring has been replaced by an imidazole ring.

Among the electron-withdrawing groups investigated in the 4(5)-position are nitro, carboxamido, and cyano. (Nitroimidazolyl)benzamides were not active in the wheat leaf antisenescence assay at 10^{-4} M. However, imidazole **13** (Q = $CONH_2$) (Parkin and Harnden, 1986) was slightly active at 10^{-5} M. Formal dehydration of **13** afforded *N*-(5-cyano-1*H*-imidazol-4-yl)benzamide (**14**) with a 100–1000-fold increase in activity (Table II).

Imidazole **14** (pSI_{50} = 7.1 ± 0.1, n = 5), the most active member of this series to date, is 10 times more active than kinetin (**1**). Substitution on the aromatic ring (benzamides **15** and **16**, X = 3- or 4-Cl) results in decreased antisenescence activity as measured by the pSI_{50} value (Table II). Unlike methylimidazolecarboxamide **11**, fluorine substitution on the aromatic ring did not affect antisenescence activity (**17**).

Recognizing that the 4(5)-cyanoimidazole was a new heterocyclic nucleus with potent cytokinin activity, other structural features known to influence cytokinin activity were examined. Other amides are exemplified by compounds **18**–**20**.

Although substitution of other groups such as *n*-butyl (**18**), furfuryl (**19**), or benzyl (**20**) for the phenyl ring of compound **14** afforded less active compounds (Table II), they are still equal in activity to some purine-based cytokinins such as *trans*-zeatin riboside (**5**; Table I).

Compounds **21** and **22** were also significantly less active than imidazole **14** in the wheat leaf antisenescence assay. The lack of chlorophyll retention activity for urea **22** was surprising since other heterocyclic ureas such as thidiazuron (**7**) (Mok et al., 1982) and *N*-(2-chloropyridinyl)-*N'*-phenylurea (**8**) (Okamoto et al., 1974) stimulate callus growth at micromolar concentrations and have pSI_{50} values of 7.9 and 6.0, respectively (Table I). Having determined that imidazole **14** was the most active member in this series of 4,5-disubstituted imidazoles as measured by pSI_{50} in the wheat leaf antisenescence bioassay, additional studies were undertaken to examine the scope of activity of these compounds as a potentially new class of cytokinin mimics using several other cytokinin bioassays (Yopp et al., 1986). Imidazole **14** has been shown to prevent protein degradation in senescing wheat leaves, to stimulate the growth of tobacco callus tissue, and to delay senescence of soybean leaf disks in our laboratories. Thus, *N*-(5-cyano-1*H*-imidazol-4-yl)benzamide (**14**) is representative of a new class of synthetic cytokinin mimics.

CONCLUSION

When structure–activity relationships for 4,5-disubstituted imidazoles, exemplified by imidazoles **11** and **14**, are examined by the wheat leaf antisenescence bioassay, imidazole **14** is consistently more active than kinetin, as measured by the pSI_{50} value. The observation of cytokinin-like activity for *N*-(5-cyano-1*H*-imidazol-4-yl)benzamide (**14**) in this and in several other cytokinin bioassays leads us to propose that these compounds represent a new class of cytokinin mimic.

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Registry No. 1, 525-79-1; **2**, 32771-64-5; **3**, 22663-55-4; **4**, 4338-47-0; **5**, 6025-53-2; **6**, 26763-63-3; **7**, 51707-55-2; **8**, 115363-82-1; **9**, 13189-13-4; **10**, 102186-88-9; **11**, 1457-60-9; **12**, 88933-82-8; **13**, 82145-23-1; **14**, 53098-81-0; **15**, 102186-66-3; **16**, 102186-67-4; **17**, 102186-58-3; **18**, 102186-64-1; **19**, 102186-79-8; **20**, 102186-80-1; **21**, 102186-63-0; **22**, 102186-60-7; **23**, 5098-11-3; $C_6H_5NH_2$, 62-53-3; 4-$FC_6H_4NH_2$, 372-19-0; C_6H_5COCl, 98-88-4; 3-ClC_6H_4COCl, 618-46-2; 4-ClC_6H_4COCl, 122-01-0; 3-FC_6H_4COCl, 1711-07-5; n-C_4H_9COCl, 638-29-9; furoyl-COCl, 527-69-5; $C_6H_5CH_2COCl$, 103-80-0; 5-methyl-4-imidazolecarbonyl chloride, 102186-98-1; benzaldehyde, 100-52-7; 5-[(phenylmethyl)imino]-1*H*-imidazole-

4-carbonitrile, 115363-81-0; phenyl isocyanate, 103-71-9; 5-cyano-4-imidazolecarbonyl chloride, 115363-80-9; 4-imidazolecarbonyl chloride, 56460-32-3.

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REVIEW

Chemistry, Analysis, Nutritional Value, and Toxicology of Tryptophan in Food. A Review

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The complex transformations of tryptophan in foods and the great diversity of derivatives formed as well as possible antinutritional and toxic manifestations are reviewed. The stability of free or protein-bound tryptophan during processing and storage depends on temperature and the presence of oxygen or other oxidizing agents, especially lipid peroxides, and radiation. In the absence of oxidizing agents, tryptophan is a stable amino acid, even in strongly basic or acidic conditions. Free or bound tryptophan is relatively stable during heat treatments such as industrial or home cooking in the presence of air or steam sterilization. Only severe treatments cause a significant degradation of this amino acid. In the presence of carbonyl compounds or/and at high temperatures, however, carboline formation occurs. Both carbolines and tryptophan-derived nitroso compounds are potential carcinogens. Tryptophan losses during food processing cannot always be monitored because of the lack of reliable analytical methods. These considerations suggest research needs for better methodology to measure tryptophan in complex foods and for new ways to prevent the formation of tryptophan-derived antinutritional and potentially toxic compounds in foods.

The nutritionally essential amino acid tryptophan was discovered by F. G. Hopkins and S. W. Cole in 1901 [for a historical account, see Curzon (1987)]. This amino acid is exceptional in its diversity of biological functions. It contributes importantly to normal growth and protein synthesis in a number of tissues (Majumdar, 1982) and regulates numerous physiological mechanisms. For example, tryptophan is the precursor of the neurotransmitter serotonin (5-hydroxytryptamine) and therefore is important in brain function (Anonymous, 1987; Wurtman, 1982). It can influence sleep in man (Pollett and Leathwood, 1983; Yuwiler et al., 1981; Trulson and Sampson, 1987) and the aging process of rats (Ooka et al., 1988). Tryptophan and some of its derivatives also alter behavior (Anonymous, 1981; Lieberman and Wurtman, 1986), particularly the regulation of the intake of food and drink (Leathwood and Ashley, 1981; Pollock and Rowland, 1981; Threatte et al., 1980). Tryptophan also serves as the in vivo precursor for the vitamin niacin (Umezawa, 1989), stimulates insulin and

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