Anticytokinin Activity of N-Phenyl- and N-Pyridylcarbamates

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A new class of non-adenylate anticytokinins, alkyl and phenyl N-phenylcarbamates and related phenyl N-pyridylcarbamates, has been developed. They have a structure that has an immediate resemblance to the non-adenylate class of cytokinins, N,N′-diphenylureas, and N-pyridyl-N′-phenylureas, and the design of the molecule was made based on insight into the bioisoteric nature between the previous, non-adenylate *s*-triazine anticytokinins and the carbamate and urea structures. The activity in terms of the I_{50} value of the most potent members, 4-fluoro- and 4-chlorophenyl N-(2-chloro-4-pyridyl)carbamates, was $0.3-0.5 \times 10^{-6}$ M when examined by the tobacco (*Nicotiana tabacum* L.) callus assay in the presence of 0.05×10^{-6} M kinetin.

Introduction

Based on the structure of N^6-substituted adenine cytokinins, five classes of anticytokinins have been developed; they are 7-substituted 3-methylpyrazolo[4,3-d]pyrimidines [1, 2], 4-substituted 7-(β-D-ribofuranosyl)- [3, 4], 4-substituted 2-methyl- [5] and 4-substituted 2-methylthiopyrrolo[2,3-d]pyrimidines [6], and 4-substituted 2-methylthiopyrido[2,3-d]pyrimidines [7]. Anticytokinins with an immediate structural resemblance to another class of cytokinins, N-alkyl-N′-phenyl- and N,N′-diphenylureas [8] are not yet known.

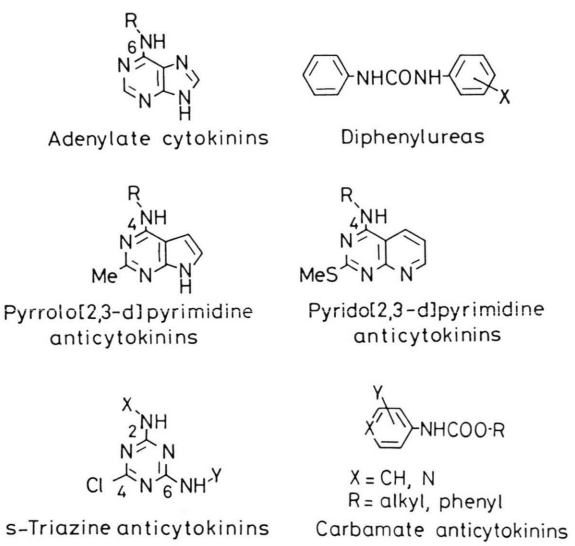

Fig. 1. Generic formulas of previously known cytokinins and anticytokinins, and new carbamate anticytokinins.

Recently, a non-adenylate class of anticytokinins, N^2-substituted 2-amino-4-chloro-6-ethylamino-*s*-triazines, has been developed by us [9]. In another place, a class of *s*-triazines is known to be an inhibitor of photosystem II electron transport in chloroplasts, though the structure and arrangement of the substituents are different from those of anticytokinins. Pertinently substituted N-phenylureas and carbamates have also been known to inhibit the electron flow by acting at the same site as that of *s*-triazines. Accordingly they are considered to be bioisosters to each other with respect to the binding to the action site, or the bioisosterism between them is fairly high, irrespective of the apparently different structures. Moreover, some N-phenylureas have cytokinin activity, as mentioned above. Therefore, the carbamate compounds are expected to behave bioisosterically to phenylureas or *s*-triazines with respect to the interaction with the cytokinin receptor as well.

The compounds thus prepared in expectation of exhibiting anticytokinin nature, rather than cytokinin activity, are N-alkyl- and N-phenylcarbamates and related N-heteroarylcarbamates. The tobacco callus assay of the set of compounds re-

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vealed that they retard the growth of cultured cells caused by kinetin, and their competitive, anticytokinin nature was confirmed kinetically by the method of Lineweaver and Burk [10]. Fig. 1 shows the generic formula of the compounds studied here together with the cytokinins and anticytokinins mentioned above.

Materials and Methods

Chemicals

Most of the compounds, **5–7, 11–18, 20, 21, 23–39,** and **41–46,** were recently reported in the literature [11] that is closely connected with this study. Other previously known compounds are **1** [12], **2, 3** [13], **4, 8–10, 19** [12], and **40** [13]. Compounds **54–56** were provided by Mitsubishi Kasei Corporation [14]. The rest were prepared for this study.

The identification of the molecular formulas, including the provided **54–56,** were made by elemental analysis for C, H, and N within the error of ±0.3%. ^{1}H NMR spectra were recorded on a JEOL PMX-60 spectrometer in $CDCl_3$ or Me_2SO-d_6 with tetramethylsilane as an internal reference.

N-Phenylcarbamates 22 and 47–51

An appropriate alcohol (mostly phenol) (11.4 mmol) was added dropwise to anhydrous benzene (30 ml) containing an appropriate phenylisocyanate (10.6 mmol) and two drops of triethylamine. The mixture was stirred for 7.5 h at room temperature, diluted with water, and extracted three times with ether. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography with *n*-hexane–ethyl acetate (80:20, v/v) or chloroform–*n*-hexane (80:20, v/v). Benzyl N-(3,4-dichlorophenyl) (**22**) (41%), m.p. 126–127 °C; 4-fluorophenyl N-(3-chlorophenyl) (**47**) (92%), m.p. 92–93 °C; 4-chlorophenyl N-(3-chlorophenyl) (**48**) (89%), m.p. 113–114 °C; 4-bromophenyl N-(3-chlorophenyl) (**49**) (88%), m.p. 118–119 °C; 4-fluorophenyl N-(3-fluorophenyl) (**50**) (41%), m.p. 112–113 °C; 4-fluorophenyl N-(3-bromophenyl) (**51**) (92%), m.p. 103–104 °C.

N-Heteroarylcarbamates 52–53, 57 and 58

To an anhydrous benzene solution of 4-amino-2-chloropyridine, 2-aminothiazole, or 3-amino-5-methylisoxazole (5.10 mmol) was added an appropriate chloroformate (6.62 mmol) and triethylamine (6.62 mmol). The mixture was stirred for 3 h at room temperature and evaporated to dryness under reduced pressure. The residue was suspended in water, and the suspension was extracted twice with chloroform. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography with chloroform–methanol (90:10, v/v). *n*-Propyl N-(2-chloro-4-pyridyl) (**52**) (36%), m.p. 140–141 °C; phenyl N-(2-chloro-4-pyridyl) (**53**) (25%), m.p. 169 °C; phenyl N-(2-thiazolyl) (**57**) (50%), m.p. 180–181 °C; phenyl N-(5-methyl-3-isoxazolyl) (**58**) (48%), m.p. 166 °C.

Tobacco callus test

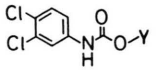
Compounds to be tested were added in different concentrations with 0.05×10^{-6} M kinetin to the basal medium as specified previously [15]. The medium was adjusted to pH 5.6 with 1 N NaOH and autoclaved at 1.0 kg/cm^2 for 15 min. Three callus pieces of about 10 mg fresh weight derived from *Nicotiana tabacum* L. var. Wisconsin No. 38 and cultured starved (on the medium containing 0.014×10^{-6} M kinetin) were implanted on the agar surface and maintained at 28 °C in darkness for 4 weeks; then the average fresh weight was found. The range of experimental error was within ± 30%. The anticytokinin activity was expressed by the I_{50} value, which is the molar concentration at which is obtained 50% of the callus growth on the medium with 0.05×10^{-6} M kinetin but without test compounds. The I_{50} and the logarithm of its reciprocal, pI_{50}, are shown in Tables I, II, and III, together with the structures of the compounds.

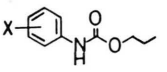
Results

The activity was tested in terms of the growth of tobacco callus derived form *Nicotiana tabacum* L. var. Wisconsin No. 38 and cultured starved [15]; the most specific and sensitive cytokinin assay known.

First examined were the activities of alkyl carbamates **1–22** in Table I in which the N-substituent

Table I. Anticytokinin activity of alkyl N-phenylcarbamates.





No.	Y	I_{50} ($\times 10^{-5}$ M)	pI_{50}	No.	Y	I_{50} ($\times 10^{-5}$ M)	pI_{50}
1	Me	21.4	3.67	**23**	H	>3.98	<4.40
2	Et	2.63	4.58	**24**	2-Cl	>3.98	<4.40
3	*n*-Pr	1.48	4.83	**25**	3-Cl	0.29	5.54
4	*n*-Bu	> 3.98	<4.40	**26**	4-Cl	2.00	4.70
5	*n*-Pent	na[a]		**27**	2-Me	1.66	4.78
6	*n*-Hx	> 3.98	<4.40	**28**	3-Me	1.15	4.94
7	Allyl	4.37	4.36	**29**	4-Me	8.32	4.08
8	Propargyl	3.55	4.45	**30**	3-OMe	>3.98	<4.40
9	*i*-Pr	2.40	4.62	**31**	4-OMe	2.95	4.53
10	*i*-Bu	3.45	4.61	**32**	4-F	7.41	4.13
11	$(CH_2)_2OMe$	> 3.98	<4.40	**33**	4-Br	1.07	4.97
12	*s*-Bu	2.34	4.63	**34**	4-NO_2	5.37	4.27
13	*t*-Bu	2.04	4.69	**35**	2,4-Cl_2	5.75	4.24
14	CH_2CHMe_3	1.41	4.85	**36**	3,5-Cl_2	0.91	5.04
15	$CH(C_2H_5)_2$	1.66	4.78	**37**	3,4,5-Cl_3	0.18	5.75
16	CH_2–*c*-Pr	1.15	4.94	**38**	3,4-Me_2	0.81	5.09
17	$CHMe_3$–*c*-Pr	1.05	4.89	**39**	2,5-Cl_2	0.21	5.68
18	*c*-Bu	1.48	4.83				
19	*c*-Pent	2.75	4.56				
20	*c*-Hx	na					
21	CH_2–*c*-Hx	na					
22	CH_2Ph	> 3.98	<4.40				

[a] na, not active.

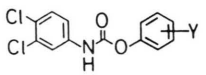
is fixed to 3,4-dichlorophenyl. In *n*-alkyl series **1–6**, *n*-propyl **3** was most potent and the activity of those having a shorter or longer *n*-alkyls was less active, suggesting that there is an optimum length for activity in the alcohol moiety. Any of the modification done by introducing a multiple bond (**7, 8**), a hetero atom (**11**), one or two branches (**9, 10, 12–15**), and a cyclic structure (**16–19**) failed to raise activity. *c*-Hexyl (**20**), cyclohexylmethyl (**21**), and benzyl (**22**) were impotent. They may be too bulky to fit the receptor. The I_{50} value of most of these alkyl carbamates was 10^{-5} M level, dozens of times less active than that of the most active member of the previous *s*-triazines [9].

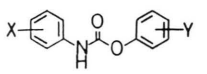
Among the *n*-propyl N-phenylcarbamate series of compounds **23–39** where the effect of aromatic substituents was examined, those with a 3-or *m*-chloro substituent (**25, 36, 37, 39**) showed I_{50} of 10^{-6} M level. Multiple chloro substitution (**36, 37, 39**) scarcely altered the activity. The activity of a 3-substituted compound tended to be higher than those of 2- and 4-counterparts in both the chloro (**24–26**) and methyl (**27–29**) series. The poorness of 3-methoxy **30** may be due to its bulkiness or its less hydrophobic nature.

We next prepared O-phenyl compounds **40–51.** The potency of the compounds with a 4-substituent tended to be generally high, at the 10^{-6} M level. The activity of unsubstituted and 2-substituted compounds was very poor. Most of the compounds were poorly soluble at concentrations higher than 10^{-5} M.

Since it has been shown by Okamoto *et al.* [16, 17] that N-(4-pyridyl)-N′-phenylureas have considerably higher activity than the corresponding N,N′-diphenylureas in cytokinin series, we prepared N-(4-pyridyl)carbamates **52–56** (Table III). The heteroaromatic moiety was fixed to 2-chloropyridine, the site of the halogen substitution corresponding to the 3-position of N-phenyl congeners. Although *n*-propylcarbamate **52** was not so potent, unsubstituted phenylcarbamate **53** was considerably so. Introduction of a chloro or fluoro substituent to the 4-position of the benzene moiety

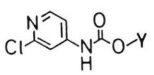
Table II. Anticytokinin activity of phenyl N-phenylcarbamates.

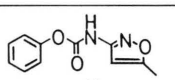
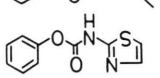




No.	Y	I_{50} ($\times 10^{-5}$ M)	pI_{50}	No.	X	Y	I_{50} ($\times 10^{-5}$ M)	pI_{50}
40	H	>3.98	<4.40	**47**	3-Cl	4-F	0.38	5.42
41	2-Me	>3.98	<4.40	**48**	3-Cl	4-Cl	0.10	6.00
42	3-Me	0.31	5.51	**49**	3-Cl	4-Br	0.27	5.57
43	4-Me	0.58	5.24	**50**	3-F	4-F	0.18	5.74
44	2-F	>3.98	<4.40	**51**	3-Br	4-F	0.18	5.74
45	3-F	0.42	5.37					
46	4-F	0.26	5.88					

Table III. Anticytokinin activity of N-pyridylcarbamates and related compounds.



No.	Y	I_{50} ($\times 10^{-5}$ M)	pI_{50}	No.	Structure	I_{50} ($\times 10^{-5}$ M)	pI_{50}
52	n-Pr	1.62	4.79				
53	Ph	0.29	5.54	**57**		0.43	5.37
54	3-Cl–Ph	0.76	5.12				
55	4-F–Ph	0.045	6.35				
56	4-Cl–Ph	0.028	6.56	**58**		2.57	4.59

of **53** gave the most active members of this class, compounds **55** and **56,** the potency being at the 10^{-7} M level and as high as that of the previous *s*-triazine [9] and adenylate pyrido[2,3-d]-pyrimidine anticytokinins [7]. 3-Chlorophenyl-carbamate (**54**) was less potent than the 4-chloro counterpart. Two other heteroaromatic compounds, **57** and **58,** were prepared. Of these, the activity of the thiazolyl derivative was as high as that of the corresponding 2-chloropyridyl (**53**).

To confirm the competitive, anticytokinin nature of the compounds, we used the method of Lineweaver and Burk [10], which has been used to examine the anticytokinin characteristic of previous adenylate [7, 18] and *s*-triazine [9] compounds. The treatment was done on compound **46,** a representative member of the class. The result is shown in Fig. 2, where the set of lines was obtained

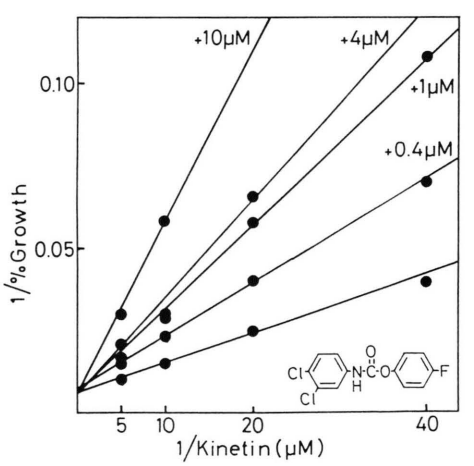


Fig. 2. Reciprocal of the growth of tobacco callus plotted as a function of the reciprocal of the concentration of kinetin alone (bottom line) and in the presence of compound **46.**

by plotting the reciprocal of the growth response against the reciprocal of the concentration of added kinetin. The fact that they had a common intercept indicates that the compound is a competitive inhibitor of cytokinins. Although this treatment was not carried out on all active compounds, the result strongly suggests the antagonistic nature of the series of compounds.

Discussion

The N-phenyl- and N-(2-chloro-4-pyridyl)-carbamates presented in this paper were shown to be competitive antagonists of cytokinins in the tobacco callus assay. That is, they are entities that fit the cytokinin receptor but do not trigger the physiological action.

Some of the structural characteristics that confer high activity seemed to be in common with those observed for the cytokinin-active N,N′-diphenylureas and N-(4-pyridyl)-N′-phenylureas [9, 17, 19]. One of them is the hydrophobic substituent at the *meta* position of one of the benzene rings in diphenylureas and at the 2-position of the 4-pyridyl moiety in pyridylureas. It has been interpreted to interact position-specifically with hydrophobic surface of the receptor [9, 19], and this coincides with the higher activity of N-(3-chlorophenyl) (**25**) and N-(3-methylphenyl) (**28**) than the 2- and 4-congeners, and also with the poor potency of the hydrophilic 3-methoxy (**30**). Another is the electron-withdrawing aromatic substituent. It has been suggested to enhance the acidity of the NH proton of urea bridge and thus the binding to the receptor *via* H-bonding. The high potency of 3-chloro **25,** 3,5- and 2,5-dichloro **36** and **39,** and 3,4,5-trichloro **37** is considered to be partly attributed to this factor, and partly to the hydrophobic effect at the 3- or *m*-position. Based on the analogy of the urea and carbamate structures, their site of electronic interaction is thought to be the NH proton of the carbamate bridge.

The *"p"*-aza function of 4-pyridyl substituent is strongly electron withdrawing (σ = 0.99) [20], and this explains the highest cytokinin activity so far known of N-(2-chloro-4-pyridyl)-N′-phenylurea [16], together with the hydrophobic as well as the electron-withdrawing *"m"*-chloro group. The same principle appears to be operating in the highly active N-(2-chloro-4-pyridyl) **55** and **56** in this anticytokinin series.

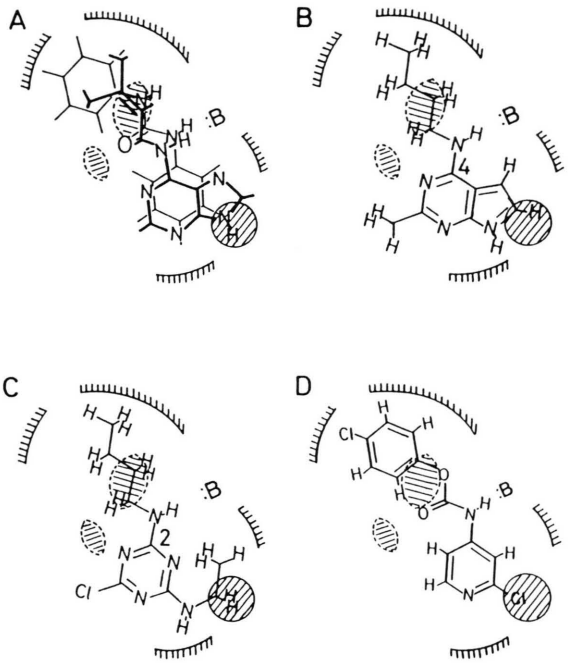


Fig. 3. Cytokinin receptor map showing the complexes with active compounds. The solid lines with a fringe represent the steric interaction sites or receptor walls on the page plane, and the ovals are those above or below it. The shaded circle shows the hydrophobic region, and :B shows the electronic interaction site or the basic group of the receptor. A, cytokinin-active 6-(3-methyl-2-butenylamino)purine (bold lines) and N,N′-diphenylurea (light lines); B, anticytokinin-active 4-(*n*-butylamino)-2-methylpyrrolo[2,3-d]pyrimidine; C, anticytokinin-active N^2-(*n*-butyl)-2-amino-4-chloro-6-ethylamino-*s*-triazine; D, anticytokinin-active 4-chlorophenyl N-(2-chloro-4-pyridyl)carbamate (**56**). In A, the ends of bars of the structures represent hydrogen atoms, and the double bonds of the aromatic rings are omitted. A, B, and C were reproduced from [9] with permission of the American Chemical Society.

Fig. 3 is the reproduction of the mode of action map of cytokinin- and anticytokinin-active compounds previously drawn after their quantitative structure-activity relationship analyses [9, 19, 21]. Cytokinin-active adenines and ureas are accommodated in A in a overlapping manner to show the structural correspondence at the site of action of the chemically different classes of compounds. Fig. 3 B and C are the accommodation of one of the adenylate anticytokinins, pyrrolo[2,3-d]pyrimidines, and anticytokinin-active *s*-triazines. The solid lines with a fringe represent the steric interaction sites or receptor walls located on the page

plane, and the ovals are those located above or below it. The shaded circle shows the hydrophobic surface where the *m*-group of ureas comes on, and :B shows the basic group of the receptor that interacts with the acidic imino proton of the ligands. These maps visually show fundamentally similar but somewhat differed modes of binding between agonists and antagonists. The previous interpretations have come up to a conclusion that the 4-substituent of pyrrolo[2,3-d]pyrimidines in B and the 2-substituent of *s*-triazines in C is closer to the upper-right wall that faces them than the 6-substituent of agonistic adenines and one of the benzene rings of the also agonistic diphenylureas in A.

Based on the structure-activity profiles observed for this series of anticytokinins, we drew Fig. 3D where the compound used as model is 4-chlorophenyl N-(2-chloro-4-pyridyl)carbamate (**56**). Thus the hydrophobic 2-chloro group is located to interact with the hydrophobic region of the receptor, and the *"p"*-aza function facilitates the H-bonding interaction of the NH group with the basic residue :B. The benzene moiety is thought to be at a closer position to the upper-right wall than that of the cytokinin-active ureas in A. In this situation, the 4-substituent of the phenol moiety may have a favorable contact with the wall at the upper left. The fundamental difference in structure of cytokinin-active ureas and anticytokinin-active carbamates is that one of the imino groups of ureas is substituted by an oxygen atom in carbamates. Thus the conversion of the activity or the binding mode from the agonistic one to antagonistic is principally attributed to this difference, although the physicochemical basis is presently unclear. Even so a further class of active compounds may be designed within the framework of Fig. 3, as was so in the development of the *s*-triazine anticytokinins [9].

The compounds of new skeletal structure may have different permeability to the site of action, different susceptibility to metabolic attack, and different affinity or selectivity to species of the cytokinin receptors in plants. Incidentally, we have found in this set of carbamates and previous *s*-triazines a novel flower-inducing activity in asparagus seedlings [11, 22], and the carbamates were much better in potency. Though the relation of this activity to the anticytokinin nature has not been clarified and is under investigation, they may be of value in deepening our understanding on various aspects of plant growth in relation with the role of cytokinins.

Acknowledgement

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[1] S. M. Hecht, R. M. Bock, R. Y. Schmitz, F. Skoog, and N. J. Leonard, Proc. Natl. Acad. Sci. U.S.A. **68,** 2608–2610 (1971).
[2] F. Skoog, R. Y. Schmitz, R. M. Bock, and S. M. Hecht, Phytochemistry **12,** 25–37 (1973).
[3] H. Iwamura, T. Ito, Z. Kumazawa, and Y. Ogawa, Biochem. Biophys. Res. Commun. **57,** 412–416 (1974).
[4] H. Iwamura, T. Ito, Z. Kumazawa, and Y. Ogawa, Phytochemistry **14,** 2317–2321 (1975).
[5] H. Iwamura, N. Masuda, K. Koshimizu, and S. Matsubara, Phytochemistry **18,** 217–222 (1979).
[6] F. Skoog, R. Y. Schmitz, S. M. Hecht, and R. B. Frye, Proc. Natl. Acad. Sci. U.S.A. **72,** 3508–3512 (1975).
[7] H. Iwamura, S. Murakami, J. Koga, S. Matsubara, and K. Koshimizu, Phytochemistry **18,** 1265–1268 (1979).
[8] M. I. Bruce and J. A. Zwar, Proc. R. Soc. London Ser. B **165,** 245–265 (1966).
[9] R. Shimizu, H. Iwamura, S. Matsubara, and T. Fujita, J. Agric. Food Chem. **37,** 236–240 (1989).
[10] H. Lineweaver and D. Burk, J. Am. Chem. Soc. **56,** 658–666 (1934).
[11] K. Yanosaka, H. Iwamura, and T. Fujita, Z. Naturforsch. **44c,** 226–232 (1989).
[12] K. Mitsutake, H. Iwamura, R. Shimizu, and T. Fujita, J. Agric. Food Chem. **34,** 725–732 (1986).
[13] S. Uwayokote, Thesis, Faculty of Agriculture, Kyoto University, Kyoto 1985.
[14] N. Kyomura, unpublished results (1989).
[15] E. M. Linsmaier and F. Skoog, Physiol. Plant. **18,** 100–127 (1965).
[16] S. Takahashi, K. Shudo, T. Okamoto, K. Yamada, and Y. Isogai, Phytochemistry **17,** 1201–1207 (1978).
[17] T. Okamoto, K. Shudo, S. Matsubara, and E. Kawachi, Chem. Pharm. Bull. (Tokyo) **29,** 3748–3750 (1981).
[18] H. Iwamura, N. Masuda, K. Koshimizu, and S. Matsubara, J. Med. Chem. **26,** 838–844 (1983).
[19] H. Iwamura, T. Fujita, S. Koyama, K. Koshimizu, and Z. Kumazawa, Phytochemistry **19,** 1309–1319 (1980).
[20] H. H. Jaffe and H. L. Jones, Adv. Heterocycle Chem. **3,** 209–261 (1964).
[21] H. Iwamura, S. Murakami, K. Koshimizu, and S. Matsubara, J. Med. Chem. **28,** 577–583 (1985).
[22] T. Abe, R. Shimizu, H. Iwamura, and T. Kameya, Physiol. Plant. **70,** 228–230 (1987).