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Original Article

Screening of Tryptophan Synthase Inhibitors as Leads of Herbicide Candidates

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We screened chemicals that inhibit tryptophan synthase for the purpose of finding the leads of herbicide candidates and found two potent inhibitors, 4-(dimethylamino)pyridine and 2-mercaptobenzimidazole; the former did not show any marked effect on rice (*Oryza sativa*) plants, whereas the latter showed considerable phytotoxicity to rice seedlings.

INTRODUCTION

Inhibitors of biosynthesis of essential amino acids can be used as herbicides with no hazardous effect on mammals. Glyphosate, sulfonylureas and imidazolinones are such herbicides. Glyphosate, for example, blocks aromatic amino acid biosynthesis, interfering with an enzyme in the shikimic acid pathway,[1] and 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase is considered as its primary site of action.[2] On the other hand, acetolactate synthase, a key enzyme for the biosynthesis of valine, leucine and isoleucine, is the target enzyme of both herbicidal sulfonylureas and imidazolinones.[3,4]

Since tryptophan is not only an essential amino acid that plays an important role in enzyme protein molecules, but also is transformed into auxin in plants, the inhibition of its biosynthesis may cause serious effects on the plants. Although tryptophan biosynthesis is blocked by inhibiting an enzyme in the shikimic acid pathway, tryptophan synthase can be a specific target of possible herbicides. This paper deals with the screening of tryptophan synthase inhibitors.

Tryptophan synthase from *Escherichia coli* has been well characterized.[5] It consists of two α-subunits and a β_2 dimer containing pyridoxal phosphate. The α-subunit catalyzes the retroaldole type cleavage of indole-3-glycerol phosphate, whereas the β_2-subunit catalyzes the condensation of indole with L-serine. The latter also catalyzes the condensation of mercaptans with L-serine.[6] In addition to a variety of heterocycles related to pyridine and indole, therefore, we examined some mercaptans for inhibitory effects on tryptophan synthase.

MATERIALS AND METHODS

1. *Tryptophan Synthase*

Purified tryptophan synthase of *Escherichia coli* was a gift from Mitsui Toatsu Chemicals, Inc. The enzyme activity of an $\alpha_2\beta_2$ subunit complex producing tryptophan from indole and L-serine was measured at pH 7.8 and 37°C by a slightly modified spectrometric method of Miles & Moriguchi's.[5]

An enzyme reaction mixture (3.0 ml) in 0.1 M potassium phosphate buffer (pH 7.8) was composed of the enzyme (0.3–0.6 mg), bovine albumin (0.5 mg/ml), pyridoxal 5-phosphate (0.05 mM), dithioerythritol (0.10 mM), indole (0.50 mM), L-serine (100.0 mM), Tris (15.0 mM) and EDTA (15–30 μM). The time-course absorbance at 290 nm was recorded on

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a Shimadzu UV-200 spectrophotometer. The reaction rate was calculated from the difference in absorbance between indole and tryptophan at 290 nm ($\varepsilon_{290\,nm}=1850\ M^{-1}\ cm^{-1}$).[5] An inhibitor solution was added to the reaction mixture, and 50% inhibitory concentrations were measured. When an inhibitor was dissolved in an organic solvent (ethanol or DMSO), the same amount of the organic solvent was added to the control.

In some experiments the initial reaction rate v was measured at various concentrations of indole [S]. The v-[S] plotting curves were obtained by applying a nonlinear least squares program (MULTI),[13] and Km and V_{max} were computed directly from the Michaelis-Menten equation.

2. Bioassay

Rice (*Oryza sativa* var. Reiho) seeds germinated with water in the dark at 25°C for four days were transplanted into sand and given water with 1/2 strength Hoagland's solution containing various concentrations of a test compound. The test compound (12.5 mg) was dissolved in 0.5 ml DMSO containing 12.5 mg Triton X-100 and the solution was diluted with diluted Hoagland's solution. Plants were cultured in a growth chamber (25°C, 60% humidity, 8 nE/cm²/sec, 12-hr photoperiod) and deionized water was added every day to compensate an evaporation loss. The plants were harvested after 14 days.

In another experiment, rice plants (two-leaf stage) grown for 14 days in a sand culture under the above-mentioned conditions, except for the absence of the test chemicals, were transplanted into sand culture watered with the Hoagland's solution containing the test chemicals. The plants were harvested after seven days. The shoot length, and fresh and dry weights of the roots and shoots of the harvested plants were measured after removing the hulls. Ten plants were used in each test.

3. Chemicals

All chemicals tested, except compounds synthesized in our laboratory as described below, were of commercial reagent grade. The synthesized compounds were purified appropriately by chromatography, recrystallization or distillation.

Synthesis of 4-(N-alkylamino)pyridines. The reactions of 1-(4-pyridyl)pyridinium chloride hydrochloride to *N*-alkylformamides (Method A)[7] or to alkylamines (Method B)[8] were applied for the preparation of the following seven compounds.

4-(N-Methyl-N-phenylamino)pyridine. A 53% yield by Method A. mp 45–47°C. ^{1}H-NMR ($CDCl_3$) δ: 3.29 (3H, s), 6.51 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz), 7.09–7.51 (5H, m), 8.17 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz).

4-(1-Pyrrolidinyl)pyridine. Method A, yield 23%. ^{1}H-NMR ($CDCl_3$) δ: 1.86–2.12 (4H, m), 3.16–3.56 (4H, m), 6.34 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz), 8.17 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz).

4-(Benzylamino)pyridine. Method A. ^{1}H-NMR ($CDCl_3$) δ: 4.29 (2H, d, $J=6$ Hz), 4.96–5.24 (1H, br, NH), 6.39 (2H, d, $J=6$ Hz), 7.26 (5H, s), 8.06 (2H, d, $J=6$ Hz).

4-(Methylamino)pyridine. Method B. mp 97°C (subl.). ^{1}H-NMR ($CDCl_3$) δ: 2.82 (3H, d, $J=6$ Hz), 4.49–5.00 (1H, br, NH), 6.40 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz), 8.14 (2H, d, $J=6$ Hz).

4-(Diethylamino)pyridine. Method B. mp 42–44°C. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (6H, t, $J=7$ Hz), 3.30 (4H, q, $J=7$ Hz), 6.40 (2H, d, $J=6$ Hz), 8.13 (2H, d, $J=6$ Hz).

4-(Isopropylamino)pyridine. Method B. mp 72–74°C. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (6H, d, $J=7$ Hz), 3.40–3.80 (1H, m), 4.56–4.89 (1H, br, NH), 6.33 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz), 8.11 (2H, d, $J=6$ Hz).

4-(Heptylamino)pyridine. Method B. mp 47–48°C. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, $J=6$ Hz), 1.04–1.72 (10H, m), 3.09 (2H, q, $J=6$ Hz), 4.94–5.16 (1H, br, NH), 6.39 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz), 8.11 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz).

4-(Dimethylaminomethyl)pyridine. This was prepared from 4-(aminomethyl)pyridine (5 ml), formic acid (9.3 ml) and 35% formaldehyde (8.6 ml) by applying the Eschweiler-Clarke reaction.[9] Yield 49%. bp 78–85°C/15 mmHg. ^{1}H-NMR ($CDCl_3$) δ: 2.24 (6H, s), 3.40 (2H, s), 7.23 (2H, d, $J=6$ Hz), 8.51 (2H, dd, $J_1=6$ Hz, $J_2=1$ Hz).

4-(2-Dimethylaminoethyl)pyridine. This was prepared by reacting 4-vinylpyridine and dimethylamine according to Phillips' method.[10]

Yield 55%. bp 94–100°C/15 mmHg. ^{1}H-NMR ($CDCl_3$) δ: 2.27 (6H, s), 2.40–2.86 (4H, m), 7.10 (2H, d, J=6 Hz), 8.46 (2H, dd, J_1=6 Hz, J_2=1 Hz).

4-Benzamidopyridine. This was prepared by benzoylating 4-aminopyridine in the presence of triethylamine, mp 205°C (subl.). ^{1}H-NMR (DMSO-d_6) δ: 7.29–8.09 (7H, m), 8.29–8.60 (2H, m), 10.53 (1H, s, NH).

2-(Methylamino)pyridine and 2-(dimethylamino)pyridine. 2-Aminopyridine was reacted with methyl iodide in the presence of sodium amide,[11] and the products were separated by silica gel column chromatography. 2-Methylaminopyridine; ^{1}H-NMR ($CDCl_3$) δ: 3.06 (6H, s), 6.38–6.56 (2H, m), 7.29–7.49 (1H, m), 8.07–8.21 (1H, m). 2-Dimethylaminopyridine; ^{1}H-NMR ($CDCl_3$) δ: 2.85 (3H, s), 4.52–5.66 (1H, br, NH), 6.21–6.61 (2H, m), 7.22–7.45 (1H, m), 7.95–8.14 (1H, m).

3-(Dimethylamino)pyridine. This was prepared by reacting 3-aminopyridine with formaldehyde and formic acid (the Eschweiler-Clarke reaction). Yield 40%, bp 98–103°C/20 mmHg. ^{1}H-NMR ($CDCl_3$) δ: 2.93 (6H, s), 6.80–7.16 (2H, m), 7.94 (1H, d, J=4 Hz), 8.09 (1H, d, J=3 Hz).

4-(Dimethylamino)quinoline. This was prepared by reacting 4-chloroquinoline with DMF.[12] Yield 88%. ^{1}H-NMR ($CDCl_3$) δ: 2.96 (6H, s), 6.66 (1H, d, J=6 Hz), 7.27–7.67 (2H, m), 8.00 (2H, d, J=9 Hz), 8.60 (1H, d, J=6 Hz).

4. *Other Methods*

Melting points were determined on an MRK apparatus and uncorrected. ^{1}H-NMR was measured with a JEOL JNM-FX 100 spectrometer at 100 MHz. Tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

1. *Screening of Tryptophan Synthase Inhibitors*

1.1 *Pyridine derivatives*

Examination of 18 pyridine derivatives indicated that an amino or hydroxy group has some effects on inhibitory activity against tryptophan synthase (Table 1). We then synthesized several aminopyridine derivatives and examined their activity. The introduction of one or two methyl groups to the amino group at the 2- or 3-position decreased the activity, whereas that at the 4-position remarkably increased it, as shown in Table 2; 4-dimethylaminopyridine was more than 100-fold as active as 4-aminopyridine. Of the *N*-mono- and disubstituted 4-aminopyridines tested, *N*,*N*-diethyl and *N*-methyl-*N*-phenyl derivatives also showed the considerably high activity (Table 3). When the amino group was separated from the pyridine ring by one or two carbon atoms the activity was lost. The endocyclic nitrogen atom possibly plays an

Table 1 Inhibitory activity of pyridine derivatives against tryptophan synthase.

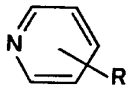
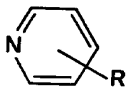
 R	I_{50} (mM)
H	> 9.2
2-NH_2	0.30
3-NH_2	0.37
4-NH_2	>10
2-OH	0.18
3-OH	0.74
4-OH	> 3
2-CH_3	>10
3-CH_3	>10
4-CH_3	>10
2,6-$(CH_3)_2$	>10
2,4,6-$(CH_3)_3$	>10
2-F	> 3.5
2-Cl	> 3.3
2-CO_2H	> 2
3-CO_2H	> 2
N^+-(4-$C_5H_4NH)Cl_2^-$	2.1
N → O	> 3

Table 2 Inhibitory activity of aminopyridine derivatives against tryptophan synthase.

 R	I_{50} (mM) Position of R		
	2	3	4
NH_2	0.30	0.37	>10
$NHCH_3$	0.40	—[a]	0.21
$N(CH_3)_2$	0.70	0.82	0.067

[a] Not tested.

Table 3 Inhibitory activity of 4-alkylaminopyridine derivatives against tryptophan synthase.

4-Substituent	I_{50} (mM)
NH_2	>10
$NHCH_3$	0.21
$NH(i\text{-}C_3H_7)$	0.15
$NH(n\text{-}C_7H_{15})$	0.14
$NH(CH_2C_6H_5)$	0.18
$N(CH_3)_2$	0.067
$N(C_2H_5)_2$	0.061
$N(CH_2)_4$	0.11
$NCH_3(C_6H_5)$	0.072
$NHCOC_6H_5$	0.30
CH_2NH_2	> 4.5
$CH_2N(CH_3)_2$	> 2.5
$(CH_2)_2N(CH_3)_2$	> 3.6

important role in enzyme inhibition, because *N,N*-dimethylaniline showed only weak activity (I_{50} = 1.0 mM).

1.2 Indoles and some other heterocycles

Table 4 shows the inhibitory activity of various heterocyclic compounds. Some indole derivatives had a certain degree of the activity. The nitrogen atom in the ring skeleton is likely to contribute to the activity: for example, 1- and 2-indanol which are similar to indole in shape having no hetero atom, showed no activity at 2 mM. Some derivatives of other nitrogen-containing heterocycles, such as benzimidazole, quinoline, imidazole, pyrimidine, and phenanthroline also showed some inhibitory activity. The activity was also found in some oxygen-containing heterocycles such as coumarin and benzodioxole.

1.3 Mercaptans

Although some aliphatic mercaptans tested did not inhibit tryptophan synthase, some heterocyclic mercaptans showed considerable inhibitory activity (Table 5). 2-Mercaptobenzimidazole was the most potent inhibitor of all the compounds tested. In comparison with the activity of benzimidazole derivatives with other functional groups (Table 4), the significance of the thiol group in inhibiting tryptophan synthase was evident. The corresponding mercaptobenzothiazole was about half as active as the benzimidazole derivative. The mercapto derivative of pyridine, however, did not show such a high activity. Details on

Table 4 Inhibitory activity of various heterocycles against tryptophan synthase.

Compound	I_{50} (mM)
Indole, 1-CH_3	0.28
6-OCH_3	0.24
2-OH	>2
2-CHO	0.14
2,3-H_2	0.51
Quinoline	0.38
4-$N(CH_3)_2$	>0.05 (28%)
8-OH	>0.6
8-OH, 2-CH_3	>0.15
2=O, 3,4-H_2	>1
Isoquinoline	1.1
Indazole	0.30
Benzimidazole	1.3
2-NH_2	0.35
2-OH	1.1
2-CF_3	2.4
Pyrrole	>7.6
Imidazole	>2.5
1-$COCH_3$	>3
1-CH_3	>5
2-C_6H_5	0.14
4-C_6H_5	1.2
2-OH	>2
Pyrazole	>10
1-C_6H_5, 3-OH	0.16
Pyrimidine	
2-NH_2	0.35
2-NH_2, 4-Cl, 6-CH_3	0.23
Pyridazine, 3,6-$(OH)_2$	>2
Phthalazine	0.75
1,10-Phenanthroline	0.12
Coumarin	0.13
7-OH	0.37
7-OH, 4-CH_3	0.43
Benzodioxole, 5-NH_2	0.52

Table 5 Inhibitory activity of mercaptans against tryptophan synthase.

Compound	I_{50} (mM)
Pyridine, 2-SH	> 0.52
Pyrimidine, 2-SH, 4-OH	0.14
Benzimidazole, 2-SH	0.045
Benzothiazole, 2-SH	0.10
Thiazole, 2-SH, 4,5-H_2	0.64
1,3,4-Thiadiazole, 2-NH_2, 5-SH	0.12
Ethane, SH	>10
Propane, 1-SH	> 5
Ethanol, 2-SH	>10
Propanoic acid, 2-SH	>10

the structure-activity relationship of mercaptobenzimidazoles will be reported in another paper.

2. *Mode of Enzyme Inhibition*

After every run of tryptophan synthase reaction in the presence of an inhibitor candidate, the reaction mixture was subjected to TLC analysis for amino acids. No amino acid besides serine and tryptophan was detected. This result indicates that the inhibitory activity of heterocyclic thiols in tryptophan synthesis was not due to their possible participation in the synthase reaction as a substrate in place of indole, although some mercaptans are known to be condensed with L-serine to give *S*-substituted cysteine by the help of this enzyme.[6]

We selected two inhibitors, 4-dimethylaminopyridine (DMP) and 2-mercaptobenzimidazole (MBI), and examined their mode of action. The v-[S] plots (Fig. 1) were obtained by changing the concentrations of indole and the inhibitors at the fixed concentrations of L-serine and pyridoxal phosphate and by computerizing them with a nonlinear least

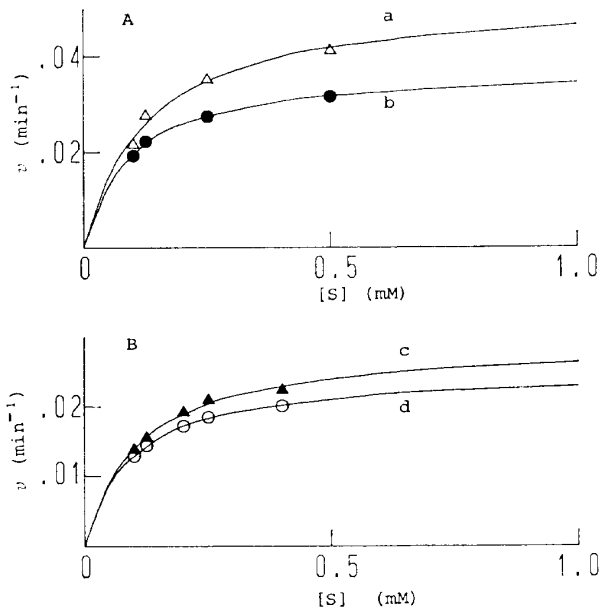


Fig. 1 v-[S] plots for tryptophan synthase inhibition by 2-mercaptobenzimidazole (MBI) (A) and 4-(dimethylamino)pyridine (DMP) (B) at a fixed concentration of L-serine (100.0 mM) and various concentrations of indole (0.1–0.5 mM).

a, no inhibitor (△); b, 0.05 mM MBI (●); c, 0.025 mM DMP (▲); d, 0.05 mM DMP (○).

Table 6 Effects of inhibitors on kinetic parameters of tryptophan synthase.

Inhibitor	Conc. (μM)	*Km* (μM)	V_{max} (μmol/min/mg protein)
non		128±26	52.37±4.32
MBI	50	90±7	37.27±1.02
DMP	25	108±11	28.99±1.12
DMP	50	93±5	24.92±0.42

Abbreviations: DMP, 4-dimethylaminopyridine; MBI, 2-mercaptobenzimidazole.

squares program.[13] These inhibitors decreased both *Km* and V_{max} (Table 6). This suggests that they are uncompetitive against indole, interfering probably with an enzyme-indole complex.

3. *Effects on Whole Plants*

The two active inhibitors were tested for effects on whole plants. Since the effects of blocking an amino acid biosynthesis may appear in the growing stage of plants more readily than in the germination stage, the tryptophan synthase inhibitors were applied to rice seedlings that had been cultured for four days after sowing and transplanted. Table 7 shows the results on the 14th day after treatment with the chemicals. Growth inhibition was observed seven days after treatment with 50-ppm MBI, whereas only little effect was found after treatment with DMP. MBI treatment caused a marked reduction in the number of roots and in the weight of both shoots and roots.

Table 7 Effects of tryptophan synthase inhibitors on rice plant growth, as % of the control.

Inhibitor	Conc. (ppm)	Shoot Length	Weight			
			Shoot		Root	
			Fresh	Dry	Fresh	Dry
DMP	5	107	100	102	92	73
	50	102	102	105	90	68
MBI	5	100	115	108	155	133
	50	37	64	85	60	42

Data were obtained 14 days after treatment of seedlings. For abbreviations see Table 6.

Table 8 Effects of tryptophan synthase inhibitors on rice plants, as % of the control.

Inhibitor	Conc. (ppm)	Shoot Length	Weight			
			Shoot		Root	
			Fresh	Dry	Fresh	Dry
DMP	50	106	117	102	88	75
	200	102	77	103	81	61
MBI	50	91	63	80	68	57
	200	89	51[a)]	74	64	55

[a)] The plants died apparently.
Plants were harvested seven days after treatment on seedlings (two-leaf stage) which had been cultured without test chemicals for 14 days. For abbreviations see Table 6.

When the chemicals were applied to rice plants which had been sand-cultured for 14 days, the plants began to wilt two days after treatment with 200-ppm MBI and apparently died about seven days after treatment without shooting third leaves. On the other hand, DMP had almost no inhibitory effect in this case, too. Table 8 shows the results obtained seven days after treatment.

In conclusion, 4-*N*,*N*-dialkylaminopyridines and 2-mercaptobenzimidazole are the potent inhibitors of tryptophan synthase as indicated by screening a variety of heterocycles and mercaptans with the enzyme from *E. coli*. The former did not have any marked effect on whole plants, whereas the latter showed considerable postemergence phytotoxicity. This type of screening with appropriate target enzymes has an apparent advantage in finding herbicidal lead compounds.

The reason of no *in vivo* effect of dimethylaminopyridine still remains unknown. This discrepancy, however, may be due to the poor pharmacodynamic ability of dimethylaminopyridine or/and the possible differences in tryptophan synthases from bacteria and plants.

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REFERENCES

1) E. G. Jaworski: *J. Agric. Food Chem.* **20**, 1195 (1972)
2) D. J. Cole: "The Herbicide Glyphosate," ed. by E. Grossbard and D. Alkinson, Butterworths, London, p. 48, 1985
3) T. B. Ray: *Plant Physiol.* **75**, 827 (1984)
4) D. L. Shaner, P. C. Anderson & M. A. Stidham: *Plant Physiol.* **76**, 545 (1984)
5) E. W. Miles & M. Moriguchi: *J. Biol. Chem.* **252**, 6594 (1977)
6) K. Soda: "Agricultural and Biological Chemistry Series 5," Vol. II, Asakura, Tokyo, p. 81, 1985
7) H. Vorbrueggen: Ger. Offen. 2,517,774; Chem. Abstr. **86**, 55293d (1977)
8) D. Jerchel & L. Jakob: *Chem. Ber.* **91**, 1266 (1958)
9) H. T. Clarke, H. B. Gillespie & S. Z. Weisshaus: *J. Am. Chem. Soc.* **55**, 4571 (1973)
10) A. P. Phillips: *J. Am. Chem. Soc.* **78**, 4441 (1956)
11) A. E. Tschitschibabin, R. A. Konowalowa & A. A. Konowalowa: *Ber. Deut. Chem. Ges.* **54**, 814 (1921)
12) N. D. Heindel & P. D. Kennewell: *J. Chem. Soc. Chem. Commun.* 38, **1969**
13) K. Yamaoka, Y. Tanigawara, T. Nakagawa & T. Uno: *J. Pharm. Dyn.* **4**, 879 (1981)

要　　約

除草活性リード化合物としてのトリプトファン合成酵素阻害剤のスクリーニング

首藤　晶，大貝真弓，江藤守総

除草活性リード化合物を見いだす目的でトリプトファン合成を阻害する化合物をスクリーニングした．そのために，*E. coli* の精製トリプトファン合成酵素を用いた．本酵素は PLP を補酵素とし，インドールと L-セリンからトリプトファンを生成するだけでなく，メルカプタンとセリンの縮合をも触媒するので，ピリジン，インドール関連化合物およびメルカプタン類を中心にスクリーニングを行なった．その結果，4-(dimethylamino)pyridine (DMP) と 2-mercaptobenzimidazole (MBI) 等に強い阻害活性が見いだされた．これらはインドールに対して不拮抗的に作用するようである．DMP は植物（イネ）生長に影響を与えなかったが，MBI は生長阻害と枯死作用を示した．