Isolation and Structure Studies of Mutagenic Principles in Amino Acid Pyrolysates

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(Received October 1, 1977)

The present paper describes the detail of the search of mutagenic principles in tars from amino acids, especially from tryptophan. A series of amino acids was pyrolyzed and was tested their mutagenicity to find that basic fractions from pyrolysates of tryptophan, ornithine, glutamic acid, serine, lysine and creatine showed potent mutagenicities to TA98 than the fractions from pyrolysates of other amino acids. Among them the active principles in the pyrolysate of tryptophan were identified as 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (designated as Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (designated as Trp-P-2).

Among several antimicrobial tar constituents, it was found that 2-amino-5-phenylpyridine, benzo[*f*]quinoline and phenanthridine also were mutagenic.

Keywords—mutagen; tryptophan; tar constituent; 5H-pyrido[4,3-*b*]indol; amino-carboline; carcinogen; aminopyridine; phenylalanine; benzo[*f*]quinoline; amino acid

Much attention has been paid for a long time that smoke condensate, charred material and other pyrolysis products are doubted to be carcinogenic.^{2,3)} Recently, Sugimura and co-workers reported that those pyrolysates showed high mutagenic activity in the Ames test.^{4,5)}

For the past decade ago, we have been searching biologically active components from various tars, including amino acid pyrolysates, and a number of antimicrobial and antihistaminic compounds has been identified.⁶⁾ The present paper describes the details on the search of mutagenic principles in the tars from amino acids, especially from tryptophan.⁷⁾

Mutagenic tests were carried out using an improved method⁸⁾ of the Ames test.⁹⁾ TA98 and TA100 strains of *Salmonella typhimurium* were kindly supplied by Dr. B.N. Ames, University of California.

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TABLE I. Mutagenic Activities of Pyrolysates of Amino Acids

Amino acid	Fraction ^{a)}	Yield (mg)	TA 100		TA 98	
			+S-9 Revertants/mg	-S-9 Revertants/mg	+S-9 Revertants/mg	-S-9 Revertants/mg
D,L-Tryptophan (10 g)	Tar	6500	2500	105	22420	16
	Basic fract.	1580	3670	118	35200	21
	A.N. fract.	3310	1670	101	250	19
L-Ornithine-HCl (5 g)	Ether sol.	359	560	k ^{b)}	4990	13
	MeOH sol.	2359	1800	k ^{b)}	7300	k ^{b)}
	Basic fract.	195	620	k ^{b)}	24950	39
	A.N. fract.	23	248	k ^{b)}	165	40
L-Glutamic acid (20 g)	Ether sol.	764	3408	53(k) ^{b)}	25515	24
	MeOH sol.	777	2020	163	3200	47
	Basic fract.	236	7030	k ^{b)}	22800	15
L-Serine (5 g)	A.N. fract.	216	295	k ^{b)}	374	k ^{b)}
	Ether sol.	198	2440	114	16783	8
	MeOH sol.	621	2600	137	20412	22
	Basic fract.	52	5640	128	19732	20
Creatinine (10 g)	A.N. fract.	10	492	161	461	31
	Tar	1785			5900	21
	Basic fract.	599			12000	24
Creatine (10 g)	A.N. fract.	130			466	27
	Tar	1785			8700	17
	Basic fract.	504			10000	32
L-Lysine-HCl (10 g)	A.N. fract.	114			479	27
	Ether sol.	345	608	k ^{b)}	4240	27
	MeOH sol.	4440	2000	k ^{b)}	5300	21
	Basic fract.	230	1360	k ^{b)}	6544	7
L-Arginine-HCl (20 g)	A.N. fract.	79	315	48	122	5
	Ether sol.	1065	1740	k ^{b)}	4763	3
	MeOH sol.	2514	1468	61(k) ^{b)}	7144	32
	Basic fract.	455	2097	29(k) ^{b)}	4309	17
L-Citrulline (4.7 g)	A.N. fract.	69	358	47(k) ^{b)}	107	22
	Ether sol.	206	1700	38(k) ^{b)}	3289	13
	MeOH sol.	1116	1900	110	4933	32
	Basic fract.	88	1436	40(k) ^{b)}	5443	16
L-Threonine (20 g)	A.N. fract.	29	377	66(k) ^{b)}	218	18
	Ether sol.	2733	1286	k ^{b)}	3062	9
	MeOH sol.	437	1440	166	3175	30
	Basic fract.	1700	835	73	3600	18
D,L-Alanine-HCl (5 g)	A.N. fract.	245	234	71	88	14
	Ether sol.	95	364	143	372	16
	MeOH sol.	1848	2120	46	2160	12
	Basic fract.	48	2420	28	2800	20
L-Cystine (10 g)	A.N. fract.	11	210	29	75	25
	Ether sol.	1081	965	132	2490	13
	MeOH sol.	552	572	190	1580	69
	Basic fract.	427	1760	47	1920	4
L-Glutamine (5 g)	A.N. fract.	304	226	118	139	4
	Ether sol.	63	1420	105	1940	30
	MeOH sol.	330	518	133	1000	25
	Basic fract.	8	5235	125	8933	97
D,L-Methionine (10 g)	A.N. fract.	5	527	k ^{b)}	355	k ^{b)}
	Ether sol.	1002	493	k ^{b)}	563	1
	MeOH sol.	536	423	80	1985	37
	Basic fract.	592	610	k ^{b)}	584	33
	A.N. fract.	64	330	66	174	17

Amino acid	Fraction	Yield (mg)	TA 100		TA 98	
			+S-9 Revertants/mg	-S-9 Revertants/mg	+S-9 Revertants/mg	-S-9 Revertants/mg
L-Cysteine.HCl (3 g)	Ether sol.	97	271	135	34	15
	MeOH sol.	1225	341	52	392	15
	Basic fract.	6	230	106	85	k ^{b)}
	A.N. fract.	64	282	148	31	19
L-Tyrosine (10 g)	Ether sol.	941	231	76	142	16
	MeOH sol.	2446	304	74	261	24
	Basic fract.	29	2380	45	2320	2
	A.N. fract.	564	172	51	57	3
L-Phenylalanine (10 g)	Tar	4800	110		103	
	Basic fract.	3200	110		200	
	A.N. fract.	700	140		230	
L-Histidine (5 g)	Ether sol.	11	1320	115	4309	18
	MeOH sol.	2224	176	107	85	25
	Basic fract.	12	536	184	1440	65
	A.N. fract.					
D,L-Asparagine (10 g)	Ether sol.	25	400	140	713	64
	MeOH sol.	343	111	101	32	32
D,L-Valine-HCl (11 g)	Ether sol.	80	146	6	24	5
	MeOH sol.	1558	168	120	39	14
	Basic fract.	20	199	67	68	17
	A.N. fract.	24	258	112	51	17
Albumine (10 g)	Tar	4000	1260	k ^{b)}	5400	k ^{b)}
	Basic fract.	800	2980	k ^{b)}	11400	k ^{b)}
	A.N. fract.	1100	260	k ^{b)}	391	k ^{b)}

a) Ether sol., MeOH sol., basic fract. and A.N. fract. (acidic-neutral fract.) were appeared in Chart 1.

b) k shows the killing effect of the fraction.

Chart 1. Separation Procedure of Acidic-neutral and Basic Fractions in Pyrolysates of Amino Acids

A series of amino acids in a flask with a thick side arm was pyrolyzed and dry-distilled by a direct flame. The distillate was separated into a basic and an acidic-neutral fraction as shown in the Chart 1. The mutagenic activity of each fraction was tested and the results were shown in the Table I. Without microsomal enzyme system (S-9 mix), each fraction did not show mutagenic activity towards both strains of TA98 and TA100. In the presence of S-9 mix, the basic fractions from most of amino acids were mutagenic but the acidic-neutral fractions were not, and TA98 was in general more sensitive than TA100. The basic fractions from pyrolysates of tryptophan, ornithine, glutamic acid, serine, lysine and creatine showed more potent mutagenicity towards TA98 than the fractions from pyrolysates of other amino

^{a)} Mutagenic activity to TA98 with S-9 mix.

Chart 2. Isolation Procedure of Trp-P-1 and Trp-P-2

acids. The pyrolysate of albumin also showed mutagenicity.

Our first attempt was to identify active principles in the pyrolysate of tryptophan, which showed the highest mutagenic activity.

D,L-Tryptophan (1.0 kg) was pyrolyzed by a direct flame to give a tar (650 g). Fractionation as shown in the Chart 2 gave 158 g of a basic mixture. The mutagenicity of the fraction to TA98 was 3520 revertants/100 μ g/plate. 50 g of the basic fraction was fractionated by a silica gel column using ethyl acetate and a mixture of ethyl acetate and methanol as elution solvents. Mutagenic substances were eluted in fractions No. [I]—32—33(1194 mg, 6000 revertants/10 μ g/plate) (Fig. 1). These two fractions were combined and subjected to alumina column chromatography using ethyl acetate and methanol. Mutagenic compounds were recovered in fractions No.[II]—32—36(160 mg, 2100 revertants/ μ g/plate) (Fig. 2). Further

Fig. 1. Silica Gel Chromatogram of the Basic Fraction

Fig. 2. Alumina Chromatogram of Fraction No. [I]—32—33

purification was carried on by converting the fraction into a water-soluble type, namely the acetate salt. Separation column was CM-Sephadex C-25, and the fractions were eluted by methanol-2N acetic acid (3:1) to give the active fractions No.[III]—11—17. Since the elution weight and the activity curve were well coincided as shown in Fig. 3, the active principles were seemed to be purified well. More efficient separation of the active components

Fig. 3. CM-Sephadex C-25 Chromatogram of Fraction No. [II]—32—36

Fig. 4. Gel Filtration of Fraction No. [III]—11—17 on Sephadex LH-20

was effected by a Sephadex LH-20 column. The methanol elution gave two main peaks with mutagenic activity maxima (Fig. 4). The first peak (fraction No. [IV]—10—11), after recrystallization from acetone-methanol, gave 5 mg of a crystalline substance which was designated as Trp-P-1 acetate,⁷⁾ whose activity was 6300 revertants/ μ g/plate. The second active peak (fraction No. [IV]—31—40) was re-chromatographed on CM-Sephadex C-25. The fractions No. [V]—12—14, after crystallization by ethyl acetate, gave 10 mg of needles, which was designated as Trp-P-2 acetate.⁷⁾ The mutagenic activity was 14000 revertants/ μ g/plate.

Recovery of the activity from the crude basic fraction was about 10 percents. A later work showed that both harman and norharman which have already been identified in the basic fraction^{6b,c)} are co-mutagenic.¹⁰⁾ Considering the activity enhancement by the presence of these co-mutagens in the crude tar, the recovery of the activity rose up to reasonable.

Trp-P-1 acetate was recrystallized from ethyl acetate to pale brown colored needles or small prisms, mp 252—262° (the crystal form changed at about 200°). Elemental analysis showed a molecular formula of $C_{15}H_{17}O_2N_3$ ($C_{13}H_{13}N_3 \cdot CH_3COOH$). Mass spectrum suggested a molecular ion of $C_{13}H_{13}N_3$ (211.1079, calcd. 211.1082). Similarly, Trp-P-2 acetate which was recrystallized from ethyl acetate and methanol, mp 248—250°, was analyzed as $C_{14}H_{19}O_4N_3$ ($C_{12}H_{11}N_3 \cdot CH_3COOH \cdot 2H_2O$), and mass spectrum suggested $C_{12}H_{11}N_3$. Ultraviolet (UV) spectrum of both compounds were almost superimposable, indicating the presence of the same chromophore. Nuclear magnetic resonance spectra (NMR), though it was taken in fairly dilute solution, showed that Trp-P-1 acetate has three methyl groups, and Trp-P-2 acetate has two methyl groups, respectively, and some aromatic hydrogens.

Fig. 5. A Perspective Drawing of the Acetate Salt of Trp-P-1 with C-C(or N, O) Bond Lengths (Å)

Fig. 6

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The structure of Trp-P-1 acetate was determined by X-ray crystallography. A crystal of size of $0.05 \times 0.1 \times 0.2$ mm, was used for X-ray experiments. It belonged to the monoclinic space group $P2_1/a$ with four molecules in a unit cell of the dimensions, $a=12.687$, $b=11.146$, $c=10.104$ Å, $\beta=103.19^\circ$. The structure was solved by the direct method using the program MULTAN and refined by the block-diagonal least-squares method. Successive least-squares calculation led the R-index of 0.04, assuming the anisotropic thermal parameters for non-hydrogen atoms and the isotropic ones for hydrogen atoms. A perspective view of the whole molecule is illustrated in Fig. 5. The chemical structure is an acetate salt of 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Fig. 6). This is quite consistent with elemental analysis, and NMR, and UV spectra.

The structure of Trp-P-2 acetate was analyzed by NMR. The absence of the higher methyl (2.11 ppm) which is present in Trp-P-1 suggests that the remaining methyl locates

TABLE II. Mutagenic Activity of Compounds isolated from Various Tar Pyrolysates

Material pyrolyzed	Compound	Revertants/100 μ g			
		TA 100		TA 98	
		+ S-9	- S-9	+ S-9	- S-9
Egg yolk	Norharman	194	99	13	27
	Harman	191	128	17	25
	1-Isobutyl- β -carboline	157	131	46	21
	1-Isopentyl- β -carboline	164	35	39	0
	1-(2-Methylbutyl)- β -carboline	197	46	62	5
	2-Butylbenzimidazole	103	89	19	8
	2-Isopentylbenzimidazole	117	75	25	5
	3-Dodecylpyridine	40/ μ l	0/ μ l	16/ μ l	3/ μ l
	3-Tetradecylpyridine	109/ μ l	79/ μ l	16/ μ l	6/ μ l
	2-Isopropyl-7-azaindole	130	107	17	17
	2-Isobutyl-7-azaindole	176	124	48	22
	2-Nonyl-5,6,7,8,-Tetrahydroquinoline	120	10	25	15
Non-fat soybean	Norharman, Harman				
	Phenanthridine	399	73	75	3
	Benzo [<i>f</i>] quinoline	690	82	87	8
Wood	1,1',3,3'-Tetraketo-2,2'-bicyclopentyl	120	102	23	10
	1,1',3,3'-Tetraketo-4-methyl-2,2'-bicyclopentyl	127	92	21	3
	Acetovanilone				
Tryptophan	Norharman, harman				
	1-Ethyl- β -carboline	125	98	38	27
d,L-Tryptophan + L-leucine	Norharman, harman, 1-ethyl- β -carboline, 1-isobutyl- β -carboline, 1-isopentyl- β -carboline				
d,L-Tryptophan + L-isoleucine	Norharman, harman, 1-ethyl- β -carboline, 1-(2-methylbutyl)- β -carboline				
L-Phenylalanine	3-Phenylpyridine		190	30	27
	2-Amino-5-phenylpyridine	2432	100	3000	11
L-Tyrosine	4,4'-Dihydroxybenzyl	147	109	18	11
	4-Hydroxybenzyl- <i>p</i> -tolyl ether	147	104	11	4
	4,4'-Dihydroxy- <i>trans</i> -stilbene				
L-Lysine	N-Phenyltyramine	204	81	31	9
	3- <i>p</i> -Hydroxyphenylpyridine	148	77	35	14
	3- <i>p</i> -Hydroxyphenyl-6-methylquinoline	204	50	44	11
	4'-Amino-2-hydroxydiphenylmethane	155	112	23	22
	4-Azafluorene	160	120	45	16
	2',3',4',5',6',7'-Hexahydroazepino-(1':2'-1:2)benzimidazole	125	109	13	5
	2-Butylbenzimidazole				
L-Arginine					
Control		110—130	100—130	16—25	8—13

at the 1 position, since the methyl group at the position should be more deshielded by the benzene ring in the molecule. The appearance of a singlet absorption at 6.35 ppm is assigned to the hydrogen at C_4 rather than the hydrogen at C_1 , because of its higher chemical shift. From these results and quite similar UV spectrum to that of Trp-P-1, the chemical structure of Trp-P-2 acetate was proposed as an acetate salt of 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Fig. 6). The structure was recently confirmed by a synthesis of Trp-P-2 acetate.¹¹⁾

Isolation studies of mutagenic pyrolysates of the other amino acids are in progress.

Several antimicrobial tar constituents have been isolated during the past decade.²⁾ Their mutagenic activities were all tested (Table II). Among them, 2-amino-5-phenylpyridine which has been isolated from D,L-phenylalanine pyrolysate^{6f)} was strongly mutagenic (3000 revertants/100 μ g/plate in TA98). Detailed data will be published in a full paper elsewhere. And benzo[*f*]quinoline and phenanthridine isolated from non-fat soy bean tar^{6c)} also showed mutagenic activity, though the activity was not so strong.

The present study suggests that there are many mutagenic principles, probably very difficult to avoid, in charred foods including hydrocarbons such as benzopyrene. The precise evaluation of mutagenicity of cooked foods and whole identification of the active components are needed for the development of less-dangerous cooking methods of daily foods. The correct estimation of the degree of carcinogenicity of synthetics such as drugs, herbicides, and other environmental substances, in comparison of power with daily cooked foods.

Experimental

Mutation Assay—Assay was carried out by modifying the method of Ames as described previously,⁸⁾ namely, S-9 was prepared from the liver of SD-rat weighing 100 g pretreated with polychlorinated biphenyl. S-9 mix is a mixture of S-9 and NADH, NADPH, ATP and glucose-6-phosphate as cofactors. Test compound dissolved in 0.1 ml dimethylsulfoxide was preincubated with 0.1 ml bacterial tester strain and 0.5 ml S-9 mix for 20 min at 37°. This mixture was added 2 ml of molten soft agar and spread over minimal glucose agar plate containing 0.1 μ mol of L-histidine and incubated for 2 days at 37°.

Pyrolysis of Amino Acids—Pyrolysis Techniques: Pyrolysis was performed over a direct gas burner flame in a Pyrex flask of enough volume with a thick side arm. Volatile product was collected in two traps cooled in ice and escaped gas was bubbled in MeOH to avoid the contamination in the air.

Fraction of Pyrolysates (Chart 1): The traps and the flask were washed with MeOH and CHCl_3 . After evaporation of the solvents, the both residues were combined and dissolved in ether. The ether solution was extracted with 1 N HCl. The 1 N HCl extract was made to alkaline with conc. NaOH and basic products were taken up in ether. Evaporation of the solvents yielded the acid-neutral fraction and the basic fraction, respectively.

Isolation of Trp-P-1 and Trp-P-2 (Chart 2)—Pyrolysis of Tryptophan: D,L-Tryptophan (250 g) was placed in a one-liter Pyrex flask with a side arm and the flask was heated on a direct flame. Volatile products were collected in two traps cooled in ice. The black tar (162 g), whose mutagenic activity to TA98 in the presence of S-9 mix was 2242 revertants per 100 μ g per plate, was obtained.

Fractionation of Pyrolysate: The tar (650 g) obtained from a total 1 kg of tryptophan (4 butches) was dissolved in 5 liters of ether and extracted two times first with one liter of 6 N HCl and then one liter of 1 N HCl, successively. The remaining ether solution contained acid and neutral products (331 g) (54 revertants/100 μ g/plate). After the aqueous solution was neutralized by Na_2CO_3 and adjusted to alkaline by 6 N NaOH, the alkaline aqueous solution saturated with NaCl was extracted with ether, to give basic products (158 g) (3520 revertants/100 μ g/plate).

Silica Gel Chromatography (Fig. 1): A portion (50 g) of the basic fraction was dissolved in AcOEt and was chromatographed over a silica gel column (4.5 $\times$ 96 cm) using AcOEt to get fractions No. [I]—1—30 of each 200 ml, and AcOEt-MeOH (7: 3) to fractions No. [I]—31—40 of each 200 ml as solvents. A large quantity of a mixture of harman and norharman was obtained from the fractions No. [I]—16—20 as crystal after evaporation of the solvent. From the fract. No. [I]—34—40, tryptamine was obtained. Mutagenic substance was eluted in fractions No. [I]—32—33 (1194 mg) (6000 revertants/100 μ g/plate).

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Alumina Chromatography (Fig. 2): The active fractions No. [I]—32—33 (969 mg) was fractionated into 37 fractions by chromatography on an alumina column (2×42 cm) using AcOEt to fractions No. [II]—1—20 of each 50 ml, AcOEt-MeOH (7:3) to fractions No. [II]—31—36 of each 50 ml and MeOH (300 ml/fractions No. [II]—37) as solvents successively, to give active fractions No. [III]—32—36 (160 mg) (2100 revertants/ μ g/plate).

CM-Sephadex C-25 Chromatography (Fig. 3): The fractions No. [II]—32—36 (160 mg) was purified by chromatography on a CM-Sephadex C-25 column (1.5×20 cm). After thoroughly washing with MeOH-H₂O (3:1, 600 ml), the active fractions No. [III]—11—17 (60 mg) (5100 revertants/ μ g/plate) was eluted with MeOH-2N AcOH (3:1).

Gel Filtration on Sephadex LH-20 (Fig. 4): Fractions No. [III]—11—17 (60 mg) was further purified by gel filtration on Sephadex LH-20 (1.5×65 cm) using MeOH as solvent and fraction of each 2.2 ml was fractionally collected. From the eluate of the first peak fractions No. [IV]—10—11, 5 mg of Trp-P-1 (6300 revertants/ μ g/plate) was isolated after recrystallization from acetone-MeOH.

CM-Sephadex C-25 Chromatography: The eluate of the second peak fractions No. [IV]—31—40 was rechromatographed on CM-Sephadex C-25 (1.5×10 cm) using MeOH-0.8N AcOH (3:1) as solvent to give Trp-P-2 (10 mg) (14000 revertants/ μ g/plate).

Physical Properties of Trp-P-1 and Trp-P-2—Trp-P-1 Acetate: Pale brown colored needles or small prisms. mp 252—262° (from AcOEt). The crystal form changed at about 200° Dragendorff reagent: positive. NMR (CD₃OD) δ : 1.18 (3H), 2.11 (3H), 2.68 (3H), 6.95—7.28 (3H, multiplet), 7.75 (1H, doublet). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3360, 3100, 1655, 1405, 1340, 1265, 1238, 925, 825, 775, 754, 743. MS m/e : 211 (M⁺), 193, 183, 169, 168, 167, 166, 142, 140, 115, 60. UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm: 245 (sh), 265, 267 (sh), 292, 305, 317. Anal. Calcd. for C₁₃H₁₃N₃·CH₃COOH: C, 66.40; H, 6.32; N, 15.49. Found: C, 66.47; H, 6.40; N, 15.04.

Trp-P-2 Acetate: Brown colored needles. mp 248—250° (from AcOEt-MeOH). Dragendorff reagent: positive. NMR (CD₃OD) δ : 1.96 (3H), 2.76 (3H), 6.35 (1H, singlet), 7.00—7.40 (3H, multiplet), 7.80 (1H, doublet). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3250, 3020, 2800—2400, 1670, 1655, 1615, 1540, 1462, 1403, 1340, 1268, 1205, 842, 734. MS m/e : 197 (M⁺), 180, 179, 170, 169, 157, 155, 127. UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm: 244 (sh), 265, 268 (sh), 290—330 (broad). UV $\lambda_{\text{max}}^{\text{MeOH}+1\text{N NaOH}}$ nm: 241, 262, 290—330 (broad). Anal. Calcd. for C₁₂H₁₁N₃·CH₃COOH·2H₂O: C, 57.32; H, 6.53; N, 14.33. Found: C, 57.71; H, 6.52; N, 14.13.