APPENDIX

Appendix 1. List of the chemicals and materials used in the study.

The chemicals used were analytical grade unless specified.

Chemicals / Materials	Source
Absolute ethanol	E. Merck, Germany
Absolute methanol	J.T. Baker Inc., U.S.A.
Ampicillin (U.S.P.)	IBI
Bacto agar	Difco Laboratories, U.S.A.
d-Biotin	Sigma chemical Co., U.S.A.
Corn oil (Commercial grade)	Sigma chemical Co., U.S.A.
Citric acid monohydrate	Fluka AG, Buchs, Switzerland
Crystal violet	E. Merck, Germany
Cysteine	Fluka Garatie, Germany
Dimethylsulfoxide (spectroscopic	E.Merck, Germany grade
Dipotassium hydrogen phosphate	Fluka AG, Buchs, Switzerland
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	E. Merck, Germany

Chemical / Material	Source
Filter paper No 1	Whatman International Ltd., England
Glucose anhydrous	Fluka AG, Buchs, Switzerland
D-Glucose 6-phosphate (monosodium salt)	Sigma Chemical Co., U.S.A.
L (+) Histidine monohydrochloride	Matheson Coleman and Bell, U.S.A.
Magnesium chloride	May and Baker Ltd., England
Magnesium sulfate ($MgSO_4.7H_2O$)	Fluka AG, Buchs, Switzerland
Millipore filter	Nihon Millipore, Kogyo,. Yonezawa, Japan
β-Naphthoflavone	Aldrich Chemical Company inc., U.S.A.
β-Nicotinamide-adenine dinucleotide reduced form (β-NADH)	Oriental Yeast Company, Japan
β-Nicotinamide-adenine dinucleotide phosphate, reduced form (β-NADPH)	Oriental Yeast Company, Japan
Oxoid nutrient broth No 2	Oxoid Ltd., England
Phenobarbital sodium (U.S.P.)	Wako Pure Chemical Industries, Ltd., Japan

Chemical / Material	Source
Potassium chloride	May and Baker Ltd., England
Sodium hydroxide	Riedel-de-Haen, Germany
Sodium ammonium hydrogen phosphate ($NaNH_4HPO_4.4H_2O$)	Fluka AG, Buchs, Switzerland
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	E.Merck, Germany
Sodium chloride	E.Merck, Germany

Appendix 2 List of instruments used in the study.

Instrument	Model	Source
Autoclave	SS-240	Tomy Seiko Co.Ltd.,Tokyo, Japan
Analytical balance	AC 100	Mettler Instrument AG., Switzerland
Circulating aspirator	WJ-15	Sibata, Japan
Colony counter	CC.3159	Anderman, England
Dispenser	Dispet TM	Nichiryo Co., Ltd., Japan
Incubator	B 5050	Heraeus, West Germany

Instrument	Model	Source
Freeze dryer	ALPHAI-6	Martin Christ GMBH & Co. KG. Germany
High Performance Liquid Chromatogrphay		LCD Analytical
UV detector (Chrom-A-Scape)		Bio-Rad
Millipore, holder	SX 001300	Millipore Corporation, U.S.A.
pH meter	701 A	Orioh Inc., U.S.A.
Polytron homogenizer	GH-6010	Kinematica, Switzerland
Refrigerator	GR-2000TG	Toshiba, Thailand
Stereomicroscope	VMZ-4SA-2W	Olympus, Japan
Superspeed centrifuge	RC2-B	Ivan Sorvall Inc., U.S.A.
Vacuum rotatory evaporator	Eyela	Tokyo Rikakikai Co., Ltd., Japan
Water bath	Type 1 No.D 7095	Yazama, Japan

Appendix 3 Induction of rat liver microsomal enzymes

The induction procedure was prepared as described by Matsushima et al., (1976). Five male Sprague-Dawley rats weighing approximately 190 g from the Animal House, Faculty of Medicine, Chiang Mai University were used. The rats were given sodium phenobarbital and 5, 6-benzoflavone (β-napthoflavone) by intraperitoneal injection each day as followings :

The rats were given drinking water ad libitum and regular complete diet.

First day, morning : a single injection of 10 mg/ml of sodium phenobarbital in saline solution, at a dosage of 30 mg per kg body weight.

Second day, morning : a single injection of 20 mg/ml of sodium phenobarbital in saline solution, at a dosage of 60 mg per kg body weight.

Third day, morning : the same dose of sodium phenobarbital as the second day.

afternoon : a single injection pf 10 mg/ml of 5, 6-benzoflavone in corn oil, at a dosage of 80 mg per kg body weight.

Fourth day, morning : the same dose of sodium phenobarbital as the second day. That evening rats were starved about 12 hours before sacrificed.

Preparation of liver homogenate fraction (S9)

On the fifth day after the induction of the liver enzymes, the rats were sacrificed by cervical dislocation and the livers were removed with sterile technique.

The freshly excised livers were placed in preweighted beaker containing approximately 1 ml of chilled 0.15 M KCl. After weighing, the livers were washed in chilled 0.15 M KCl for several time. The washed livers were transferred to a beaker containing 3 volumes of chilled 0.15 M KCl (3 ml/g wet liver). and were minced with sterile scissors, and homogenized with a Polytron Homogenizer. The homogenate was centrifuged for 10 minutes at 9,000 g, the supernatant; S9 fraction) was saved. The freshly prepared S9 fraction was distributed in 1-2 ml portions in cryogenic vials (Wheaton), quickly freeze and stored immediately at -80 ^{o}C. The protein concentration in S9 fraction was determined by the method of Lowry et al. (1951) and was approximately 30 mg/ml, which was constant from preparation to preparation. One ml of S9 fraction contained microsomes from about 250 mg of wet liver.

As required for mutation assay, a sufficient S9 fraction was thawed at room temperature and S9 mix was prepared as soon as the S9 had thawed and the S9 mix was kept in ice. The remaining S9 fraction was discarded.

The S 9 mix (rat liver microsomal enzymes + cofactors)

S9 mix was freshly prepared each day and usually kept in ice. The components of 1 ml the standard S9 mix were

0.2 M Sodium phosphate buffer, pH 7.4	0.5 ml.
0.4 M $MgCl_2$ - 1.65 M KCl	0.02 ml.
1 M Glucose-6-phosphate	0.005 ml.
0.1 M NADPH	0.04 ml.
0.1 M NADH	0.295 ml.
Sterile distilled H_2O	2.295 ml.
Rat liver S9 fraction	0.1 ml.

The ingredients should be added in the order and should be chilled. Any left S9 fraction or S9 mix was discarded.

Appendix 4 Preparation of some reagents

Preparation of minimal glucose agar plate

The components of 1000 ml minimal glucose agar medium were

Bacto-Difco agar	15	g
Distilled water	850	ml
10xVogel-Bonner medium E	100	ml
40% Glucose	50	ml

The ingredients should be autoclaved separately, when the solution has cooled slightly, added together, mixed well and poured 30 ml into each plate.

The components of 1,000 ml the Vogel-Bonner medium E (ten-fold solution) (Vogel and Bonner, 1959) are

$MgSO_4.7H_2O$	2	g
Citric acid. H_2O	20	g
K_2HPO_4	100	g
$NaNH_4HPO_4.4H_2O$	35	g

Preparation of top agar containing histidine and biotin

A: The components of 100 ml top agar

Bacto-Difco agar	0.6	g
NaCl	0.5	g
Distilled water	100	ml

The solution was steriled by autoclave at 1 lb, 120 ^{o}C, 20 min

B: The components of 100 ml 0.5 mM histidine/biotin

Ingredient	per liter	
D-Biotin	124	mg
L-Histidine HCl H_2O	105	mg
Distilled H_2O	100	ml

Dissolve histidine and biotin by stirring in water. Sterilize by filteration through membrane filter (0.22 um pore size).

Depending on numbers of plate for each test, calculate a total volume of top-agar required (according to the amount of top agar used = 2 ml per plate). The Histidine/biotin solution will be added to the Top agar before starting the mutagenic experiment by the ratio of 10 ml of solution B : 100 ml solution A.

Name : Miss Nattaka Lomsri

Date of birth : 25 November 1962

Education

March, 1979 Certificate of Mathayom Suksa III
Regina Coeli School, Chiang Mai

March, 1981 Certificate of Mathayom Suksa V
Montfort College, Chiang Mai

May, 1986 Bachelor of Science (Biology)
Faculty of Sceince, Ramkamhaeng University, Bangkok

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