CHEMISTRY OF ENZYME INHIBITORS OF MICROBIAL ORIGIN

HAMAO UMEZAWA

Institute of Microbial Chemistry and Department of Antibiotics, National Institute of Health, Tokyo, Japan

ABSTRACT

Study of antibiotics has furnished interesting materials to chemistry of natural products. I initiated the screening study of enzyme inhibitors produced by microorganisms and isolated leupeptin and antipain inhibiting trypsin and papain, chymostatin inhibiting chymotrypsin, pepstatin inhibiting pepsin, panosialin inhibiting sialidases, oudenone inhibiting tyrosine hydroxylase, dopastin inhibiting dopamine β-hydroxylase, aquayamycin and chrothiomycin inhibiting tyrosine hydroxylase and dopamine β-hydroxylase. Structures and syntheses of most of these compounds have been studied. I also found dopamine β-hydroxylase-inhibiting activity of fusaric acid and oosponol, and xanthine-oxidase inhibiting activity of 5-formyluracil which were produced by micro-organisms. Chemical study of enzyme inhibitors has given useful information on the structure/activity relation. The effect of pepstatin on stomach ulcer, and the hypotensive effect of oudenone and fusaric acid have been observed clinically. Enzyme inhibitors produced by microorganisms are the most valuable new area extended from antibiotics and will furnish new materials interesting in chemistry. biosynthesis, pharmacology, and utility.

Research on antibiotics has contributed to the chemistry of natural products, furnishing materials of interesting structures, chemical syntheses, biosyntheses and of interesting bioactivity. However, because the field has been so extensively studied, the probability of discovering new antibiotics, especially new and useful agents, is now significantly reduced. In these circumstances, it is felt that the most valuable contribution would be to extend these studies to a new and potentially more fruitful area. In 1965, in the hope of exploiting a new area, I initiated the screening of enzyme inhibitors in culture filtrates of microorganisms, with the collaboration of Doctors Takeuchi, Aoyagi, Kondo, Maeda, Hamada, Takita and others in my laboratories, Doctor Nagatsu, Department of Biochemistry, Dental School of Aichi Gakuin University, Nagoya, and Doctor Ohno, Basic Research Laboratories, Toray Industries.

At present, the structures of small molecular compounds can be determined rapidly, and their syntheses accomplished in a short period of time. This recent progress in the chemistry of natural products through the use of physical methods is a driving force in the study of enzyme inhibitors in microbial culture filtrates. The structures elucidated stimulate the study of

P.A.C.—33-1—F

the mechanism of inhibition and structure-activity relationships. To continue the study of natural products exhibiting biological activity is far easier today than it was ten years ago. As shown in this paper, inhibitors which we found in microbial culture filtrates are of small molecular nature, and their structures could be rapidly determined.

Specific enzyme inhibitors are biochemical tools useful in the analysis of biological functions and diseases. If a biological function is inhibited or interfered with by a specific inhibitor, then it is clear that the enzyme reaction inhibited is in some way related to this function. If a disease symptom is removed or changed by a specific enzyme inhibitor, then the enzyme reaction inhibited must bear a direct or indirect relationship to this disease process. Moreover, some specific enzyme inhibitors have potential utility in the treatment of diseases.

As reported in this paper, it is now certain that microorganisms produce inhibitors of various enzymes. However, when I initiated this study, I had to speculate on the possible existence of such compounds from our experiences in the study of antibiotics and from studies of enzyme inhibitors of animal and plant origin. Naturally occurring enzyme inhibitors have been searched for in tissues of animals and plants. As already reviewed[1], animal and plant tissues generally contain inhibitors of proteolytic enzymes. Inhibitors of enzymes other than proteolytic enzymes have also been searched for in animal and plant tissues. The inhibitors of animal and plant origin are peptides of molecular weight above 5000 with a few exceptions[2–4].

Table 1. Inhibitors of proteolytic enzymes and their origin

Inhibitors	Species
Leupeptin	*S. roseus*, *S. roseochromogenes*, *S. lavandulae*, *S. albireticuli*, *S. thioluteus*, *S. chartreusis*, other more than 11 species of *Actinomycetes*
Antipain	*S. michigaenis*, *S. yokosukaensis*, *A. violascens* etc.
Chymostatin	*S. hygroscopicus*, *S. lavendulae* etc.
Pepstatin	*S. testaceus*, *S. argenteolus*, etc.

It seems that studies of enzyme inhibitors of animal and plant origin arose from the concept of the coexistence of inhibitors in tissues with enzymes inhibited. It suggests the possible existence of inhibitors of proteolytic enzymes in culture filtrates of microorganisms. Apart from microbial cells proteolytic enzymes hydrolyse organic nitrogen compounds in the medium, but they are thought to be harmful to cells. This suggests the probable production of protease inhibitors by microorganisms. Such inhibitors are thought to be produced widely by various species of the same genus. As shown in *Table 1*, the results of our studies, especially on leupeptin which inhibits trypsin and papain, seem to concur with these concepts, because leupeptin is produced by more than 17 species of actinomycetes.

Based on the foregoing, even before initiating the search for enzyme

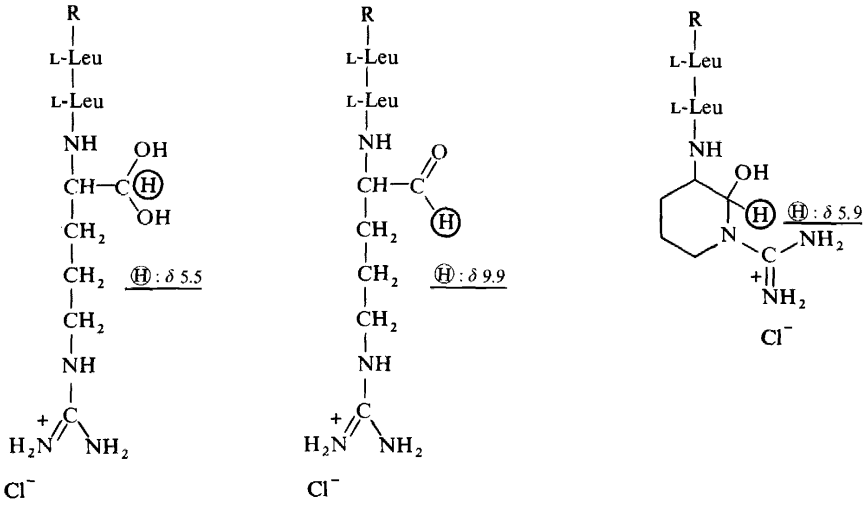
inhibitors in fermented broth, it was felt reasonable to assume that we would be successful in the isolation of inhibitors of proteolytic enzymes.

We were also challenged to search for inhibitors of enzymes which are involved in biological functions of animals. The possible existence of such inhibitors in microorganisms was supported only by the fact that microorganisms are capable of producing innumerable compounds of various types of structures. In this case, it is thought that we should screen culture filtrates, not only of actinomycetes, bacteria and fungi but also of mushroom cultures. With the collaboration of Doctor Tanabe, Director of Research Laboratories, Takara Shuzo Company in Osaka, we were able to study mushroom cultures. We succeeded in finding inhibitors of tyrosine hydroxylase and dopamine β-hydroxylase and we extended the study to various other enzymes. In this paper, I will report on the chemistry of inhibitors of proteolytic enzymes, tyrosine hydroxylase, dopamine β-hydroxylase and some other enzymes with a brief description of their activity.

The screening of actinomycetes culture filtrates by testing for activity to inhibit plasmin or trypsin led to the discovery of leupeptin[5,6]. Leupeptin is the name of a group of streptomyces products inhibiting proteases and includes acetyl or propionyl-L-leucyl-L-leucyl-argininal and those which contain L-isoleucine or L-valine instead of L-leucine. For convenience, we call acetyl-L-leucyl-L-leucyl-argininal leupeptin Ac-LL and propionyl-L-leucyl-L-leucyl-argininal leupeptin Pr-LL. In general, leupeptin Ac-LL and leupeptin Pr-LL are produced in cultured broth together with small amounts of other leupeptins. It caused difficulty in the purification. This difficulty was solved by studies of leupeptin produced in synthetic media. In a synthetic medium containing leucine, arginine and glycine, glucose, starch and salts, leupeptin Ac-LL and Pr-LL were produced without production of other leupeptins. Leupeptin can be extracted by an ion exchange resin process, using porous carboxylic resin. Two leupeptins containing acetyl or propionyl were separated by silicic acid column chromatography of their dibutyl acetals, using butanol–butyl acetate–acetic acid–water in 4:8:1:1. In thin layer chromatography, using silica gel G and butanol–butyl acetate–acetic acid–water in 4:2:1:1, even a single leupeptin showed two spots: acetyl-L-leucyl-L-leucyl-argininal 0.35 and 0.45; propionyl-L-leucyl-L-leucyl-argininal 0.45 and 0.50. Before confirmation of this fact, it caused difficulty in the structure determination. The signal of the proton on aldehyde carbon in n.m.r. indicates that a leupeptin is present in the hydrated form and in the cyclic form as shown in *Figure 1*. These two forms suggest the possible appearance of two spots on thin layer chromatography[7]. N^{α}-acetyl-argininal also shows two spots.

The structure of leupeptin was determined by degradation of leupeptin, leupeptic acid and leupeptinol and their syntheses[8,9]. Arginine obtained by hydrolysis of leupeptin acid was racemic. In studying the activity of synthetic leupeptin Ac-LL, it was proved that the L-argininal form is active and the D-argininal form is not active[7,9]. Leupeptin acid, leupeptinol and the dibutyl acetal of leupeptin are not active, and therefore the aldehyde of L-argininal moiety is thought to be essential for the antiprotease activity.

Leupeptin analogues[7,9] have been synthesized and their activities are shown in *Table 2*. N^{α}-acetylargininal is not active. Acetylleucyl-L-argininal



Leupeptin Ac-LL: R = CH_3CO
Leupeptin Pr-LL: R = CH_3CH_2CO

Figure 1.

is active. Acetyl-L-isoleucyl-L-leucyl-L-argininal and acetyl-L-valyl-L-leucyl-L-argininal were found to be more active than natural leupeptins in inhibiting plasmin.

Leupeptin inhibits plasmin and bradykinin formation[10]. If leupeptin ointment is applied immediately after a burn, it suppresses pain and blister formation. This effect suggests that kinin has a predominant role in causing pain and blistering in burns.

Table 2. Inhibition of proteases by leupeptin and the relating peptides

Compounds*	ID_{50} (μg/ml)		
	Plasmin†	Papain‡	Thrombokinase
N^{α}-Ac-Argal	>333	—	—
Ac-Leu-Argal	8	0.3	38
Pr-Leu-Argal	8	—	—
Ac-Ileu-Argal	33	—	—
Ac-Leu-Leu-Argal	9	0.8	14
Pr-Leu-Leu-Argal	8	0.5	14
Ac-Ileu-Leu-Argal	2	2.0	11
Ac-Val-Leu-Argal	2	1.7	9
Ac-Ileu-Ileu-Argal	35	6.5	—
Ac-Ileu-Val-Argal	314	15.5	—

Abbreviations: Ac: acetyl, Pr: propionyl, Leu: L-leucyl, Ileu: L-isoleucyl, Val: L-valyl, Argal: argininal
* All amino acids and argininal have L configuration.
† Fibrinogenolysis by plasmin.
‡ Caseinolysis by papain.

In testing for the ability of culture filtrates of actinomycetes to inhibit papain, we discovered another inhibitor named antipain. S. Umezawa and his associates, Department of Chemistry, Institute of Technology, Keio University found a new compound in their studies on Sakaguchi positive metabolites of actinomycetes. Antipain was identical with this guanidine peptide. S. Umezawa *et al.* proposed the structure in *Figure 2*. Structurally antipain is related to leupeptin in its argininal moiety. As the activities are shown in *Table 3*, antipain and leupeptin both inhibit trypsin and papain; however, the former is weaker in inhibiting plasmin than the latter.

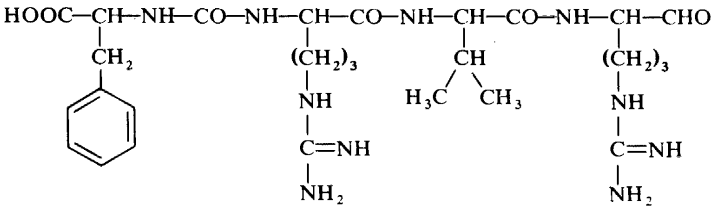


Figure 2. Antipain.

Our screening studies, utilizing the determination of antichymotrypsin activity, resulted in the isolation of an inhibitor named chymostatin from seven strains of actinomycetes[11]. Chymostatin was crystallized, but amino acid analysis suggested that it was a mixture of peptides. As shown in *Table 3*, chymostatin inhibits chymotrypsin and weakly papain but not trypsin.

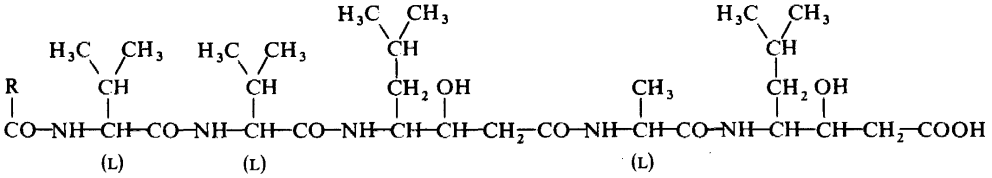
As discussed previously, a specific enzyme inhibitor is useful for the analysis of pathogenic phenomena and also perhaps for the treatment of certain diseases. Under this aspect, among protease inhibitors, a specific pepsin inhibitor is most valuable, because no compound exhibiting such activity has been available before. By testing for antipepsin activity of actinomycetes culture filtrates we found pepstatin[12, 13]. As shown in *Table 3*, pepstatin inhibits specifically acid proteases and cathepsin D.

Extraction of a culture filtrate with butanol, concentration under reduced pressure and decolorization with carbon yielded pepstatin crystals. The structure of this pepstatin was determined by isolating isovaleric acid, L-valine (2), L-alanine (1) and 4-amino-3-hydroxy-6-methylheptanoic acid (2) from the hydrolysate in the molar ratio shown in parentheses and by the high resolution mass spectroscopy of permethylated pepstatin methyl ester. The structure of this pepstatin in *Figure 3* (R = isovaleryl) suggested the possibility of producing compounds of this type which differ from one another in the fatty acid moiety or in the amino acid moiety. Actually, another pepstatin containing an *n*-caproyl group was isolated. Gas chromatographic analysis of hydrolysates of various pepstatins indicated the presence of more than five pepstatins which differ from each other in the fatty acid moiety. After we reported the isolation and structure determinations, Murao[14] reported the isolation of a pepstatin containing an acetyl group.

Table 3. Effect of leupeptin, chymostatin, antipain and pepstatin against various proteases

Enzymes	Substrates	ID_{50} (μg/ml)			
		Leupeptin	Chymostatin	Antipain	Pepstatin
Thrombokinase	Plasma	15	>250	20	>250
Thrombin	TAME*	10000	>250	>250	>250
Plasmin	Fibrinogen	8	>250	93	>250
Trypsin	Casein	2	>250	0.26	>250
Papain	Casein	0.5	7.5	0.16	>250
Kallikrein	BAEE†	75	>250	>250	>250
α-Chymotrypsin	Casein	>500	0.15	>250	>250
β, γ and δ-Chy-try.	Casein	>500	0.15	>250	>250
Pepsin	Casein	>500	>250	>250	0.01
	Haemoglobin	>500	>250	>250	0.0031
Proctase A	Casein	>250	>250	>250	>250
Proctase B	Casein	>250	26.5	190	0.0072
Cathepsin A	Cb-Glut-Tyr‡	1680	62.5	—	>125
Cathepsin B	BAA§	0.44	2.6	—	>125
Cathepsin D	Haemoglobin	109	49.0	—	0.011

* N^a-(*p*-toluenesulphonyl)-L-arginine methyl ester HCl.
† N^a-benzoyl-L-arginine ethyl ester HCl.
‡ Carbobenzoxy-L-glutamyl-L-tyrosine.
§ N^a-benzoyl-L-arginine amide HCl.



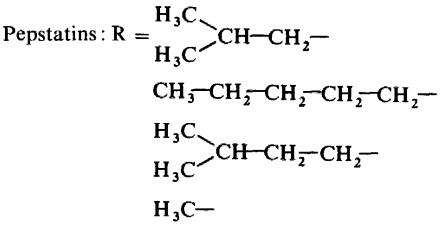


Figure 3.

The most detailed chemical and biological studies have been performed on the pepstatin containing the isovaleryl group[15]. The free carboxyl group is not essential for its antipepsin activity. This is in accord with the effect exhibited under acid conditions for the pepsin reaction. Methyl and ethyl esters of pepstatin show the same or a little higher activity than pepstatin, and the amide shows almost the same or slightly lower activity than pepstatin. At least one of two hydroxyl groups of pepstatin seems to be involved in the activity. Treatment of pepstatin with acetic anhydride in pyridine gives dehydropepstatin and dehydroacetylpepstatin. In dehydropepstatin one of the hydroxyl groups, probably the hydroxyl group of the terminal 4-amino-3-hydroxy-6-methylheptanoic acid moiety, is dehydrated. Dehydropepstatin showed 60 per cent activity of pepstatin and dehydroacetylpepstatin about one per cent activity. Isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoic acid showed the antipepsin activity at 10 μg/ml, that is, 0.1 per cent activity of pepstatin. These results suggest that this new amino acid is the most important moiety for antipepsin activity.

The strong binding of pepstatin with pepsin has been proved by two experiments. As shown by the experiment in *Figure 4*, considering the contamination of inactive pepsin in the pepsin employed, pepstatin binds strongly with pepsin in equimolar ratio. The dissociation constant is less than 10^{-9} M. The binding is not of covalent nature. From the pepstatin and pepsin mixture, pepstatin can be extracted into butanol at acid pH. If the mixture is neutralized and pepsin is inactivated, pepstatin recovers.

In experimental rat stomach ulcer, that is, in the ulcer caused by pylorus ligation, pepstatin shows a strong protective effect indicating that pepsin is the main cause of this experimental rat ulcer. The clinical results have shown that administration of one capsule containing 50 mg of pepstatin gives enough concentration to inhibit peptic activity of gastric juice at least for one hour[15]. The therapeutic effect of pepstatin on stomach ulcer has been confirmed by clinical studies in the last two years.

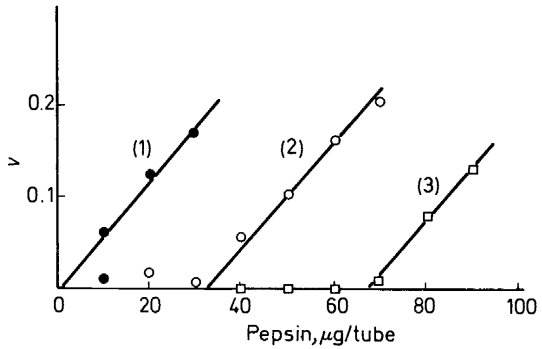


Figure 4. (1): A tube contains 0.17 ml of 2 mM acetyl-L-phenyl-L-diiodotyrosine in 4 mM caustic soda, 3.03 ml of 0.1 N hydrochloric acid, 0.2 ml of 0.001 N hydrochloric acid containing varied amounts of pepsin. (2): (1) with 0.5 μg of pepstatin per tube. (3): (1) with 1.0 μg of pepstatin per tube. *v*: Initial velocity of hydrolysis: after 10 min at 37° hydrolysis was determined by ninhydrin method, reading the absorbance at 570 mμ.

In the screening studies, testing for activity of actinomycetes culture filtrates against virus sialidases, we found a group of compounds named panosialin[16] in culture filtrates of *Streptomyces pseudoverticillus* and *Streptomyces rimosus forma panosialinus.* Panosialin is extracted into butanol from the culture filtrate and crystallized from water as the potassium salt. Panosialin sodium is soluble in water, but the potassium salt is insoluble and the potassium salt is easily crystallized from water by addition of potassium ion. The structure studies have shown that potassium panosialin crystals were a mixture of 5-isopentadecylbenzene-1,3-disulphate, 5-*n*-pentadecylbenzene-1,3-disulphate, 5-isohexadecylbenzene-1,3-disulphate and small amounts of other 5-alkylbenzene-1,3-disulphates (*Figure 5*). The dimethyl ether of 5-*n*-pentadecylresorcinol obtained by hydrolysis was

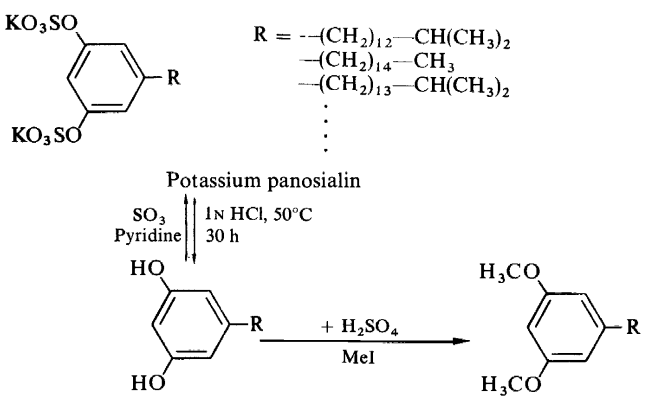


Figure 5.

identical with that synthesized. The structure suggests that panosialin molecules might aggregate in the aqueous solution depending on the hydrophobic interaction. Panosialin is not easily dialysed, and aggregation is also suggested by ultracentrifugation. It can be surmised that though panosialin is a small molecular compound, it behaves like a polyanionic compound. Thus, panosialin inhibits not only sialidases but also trypsin, plasmin and pepsin, if panosialin is incubated with these enzymes before addition of the substrates.

As described above, we have succeeded in finding protease inhibitors in culture filtrates of actinomycetes. We also screened culture filtrates of various microorganisms, testing for activity to inhibit enzyme reactions involved in biological functions in animals. In animals, norepinephrine, which is the sympathetic neurotransmitter is synthesized from tyrosine through the route shown in *Figure 6*. Tyrosine hydroxylase is involved in the rate-limiting step of the norepinephrine biosynthesis, and dopamine β-hydroxylase in the last step of the biosynthesis exists not only in cytoplasm but also in the norepinephrine particle where tyrosine hydroxylase is not present. Thus, we screened for the ability of culture filtrates to inhibit tyrosine hydroxylase and dopamine β-hydroxylase.

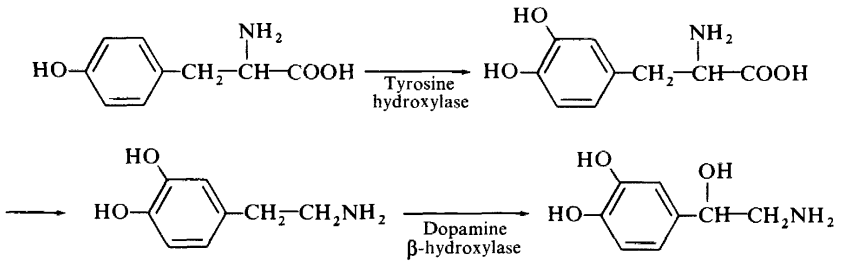


Figure 6.

From a culture filtrate of a mushroom classified as *Oudemansiella radicata*, a specific tyrosine hydroxylase inhibitor was isolated by extraction with an organic solvent at acid pH and reextraction from the solvent with water at neutral pH, and recrystallized from warm hexane[18]. The molecular formula of $C_{12}H_{16}O_3$ was obtained by high resolution mass spectroscopy. It had acidic properties with a pK_a' of 4.1. The partial structure, $CH_3CH_2CH(O)CH_2CH_2C$, was obtained by n.m.r. through the application of the double and triple resonance technique. Ozonolysis afforded succinic acid and γ-propylbutyrolactone. Ohno noticed that the unique signals of the ABXY system at δ 3.19 and δ 3.57 were due to a large geminal coupling of two protons of methylene adjacent to an exo- or endo- double bond of a five- or six-membered ring, leading to the structure in *Figure 7*[19]. This structure was proved by hydrolysis in water at 150°, affording 1,3-cyclopentanedione and γ-propylbutyrolactone. The S configuration could be assigned to the lactone by the Hudson–Klyne lactone rule.

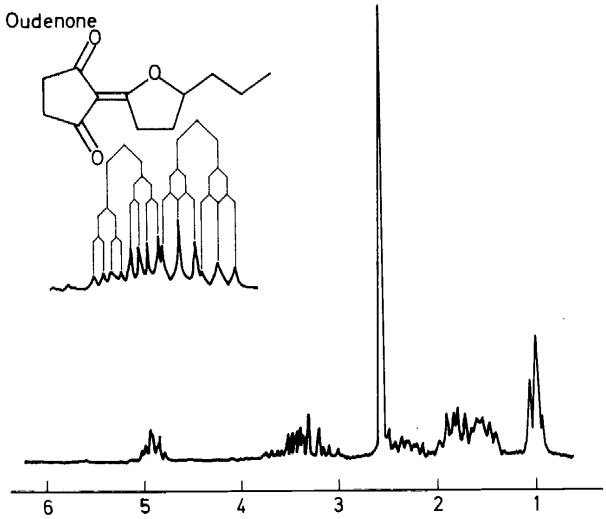
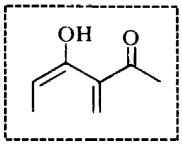


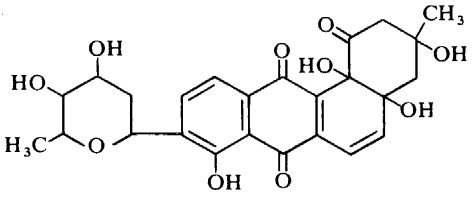
Figure 7.

Oudenone was synthesized by Ohno *et al.* by condensation of 2-acetyl-1,3-cyclopentanedione with furfural in the presence of morpholine followed by hydrogenation over platinum in the presence of one mole equivalent of sodium hydroxide. A most interesting fact is that the dl-oudenone synthesized has the same activity as natural l-oudenone. It was further confirmed that each optical isomer has the same activity to inhibit tyrosine hydroxylase.

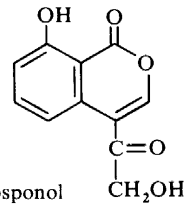
Oudenone-inhibiting biosynthesis of norepinephrine shows a hypotensive effect, especially a strong effect against hypertension in spontaneously hypertensive rats. Thus, it was shown that oudenone is a useful tool for the analysis of biochemistry of hypertension. The fact that synthetic dl- and d-oudenone also showed a hypotensive effect suggested the possibility of development of oudenone-related compounds exhibiting a stronger activity than oudeone. Synthesis of oudenone-related compounds will be reported by Ohno *et al.* in the near future.

In screening culture filtrates of actinomycetes for activity to inhibit tyrosine hydroxylase, we isolated many coloured compounds[20,21]. Among them the structure of aquayamycin has been elucidated by Sezaki and Ohno[22] (*Figure 8*). Aquayamycin has antibacterial and antitumour activity but is quite toxic. Therefore, the action of this compound is not specific to tyrosine hydroxylase. However, the partial structure surrounded by the dotted line in *Figure 8* is interesting, because these types of compounds have the ability to inhibit tyrosine hydroxylase or dopamine β-hydroxylase. Deoxyfrenolicin (*Figure 8*), an antibiotic produced by actinomycetes, was reported by Taylor *et al.*[23] to inhibit tyrosine hydroxylase. We observed that quercetin inhibited dopamine β-hydroxylase and rutin inhibited tyrosine hydroxylase.

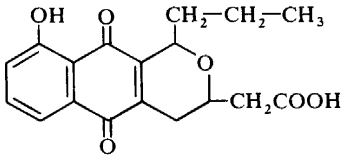




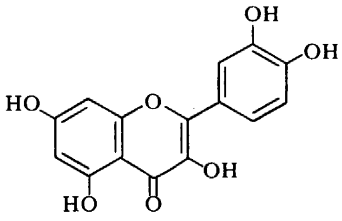
Aquayamycin



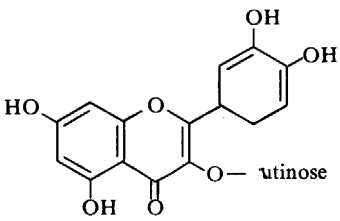
Oosponol



Deoxyfrenolicin



Quercetin



Rutin

Figure 8.

A compound discovered in our studies by testing the effect of culture filtrates of a mushroom was found to be identical with oosponol which had been described as a metabolite of a fungus by Yamamoto *et al.*[24] and synthesized by Sakan *et al.*[25] recently. Oosponol inhibits dopamine β-hydroxylase: 50 per cent inhibition at 3.4×10^{-4} M. We found that it exhibits a hypotensive effect in spontaneously hypertensive rats. Oosponol is an interesting inhibitor of dopamine β-hydroxylase; however, it is strongly allergenic. It is not irritating to subjects on first exposure, but it causes severe skin rash, severe bronchitis and pneumonia in persons after several contacts.

In our screening studies, from fungi classified as *Fusarium* spp., we isolated a product showing strong inhibition of dopamine β-hydroxylase[26] which was identical with fusaric acid (fusarinic acid), 5-butylpicolinic acid, which had been discovered by Yabuta *et al.* in 1934[27] as a fungus metabolite. We found that fusaric acid inhibited norepinephrine biosynthesis and showed a hypotensive effect. We synthesized homologues in the sidechain of fusaric

acid, that is, 5-alkylpicolinic acids, and tested for their activity to inhibit dopamine β-hydroxylase and to lower the blood pressure in normal rabbits. Results are shown in *Figure 9*[28]. All 5-alkylpicolinic acids have both the ability to inhibit dopamine β-hydroxylase and to lower the blood pressure. The strengths of both activities are parallel as shown by the parallel lines of these activities in *Figure 9*. Among the 5-alkylpicolinic acids, 5-butyl and 5-pentylpicolinic acids are the strongest in both activities. Their calcium salts have been studied clinically and the hypotensive effect has been confirmed. An interesting finding is their effect on alcoholism. Dr Ogata[29], Department of Psychiatrics, Sapporo City Medical School, found that after daily oral administration of 200 to 600 mg for one to two weeks, patients who were addicted to alcohol became sensitive to alcohol, reducing their capacity to drink it. This observation indicates the possible involvement of dopamine β-hydroxylase in alcoholism.

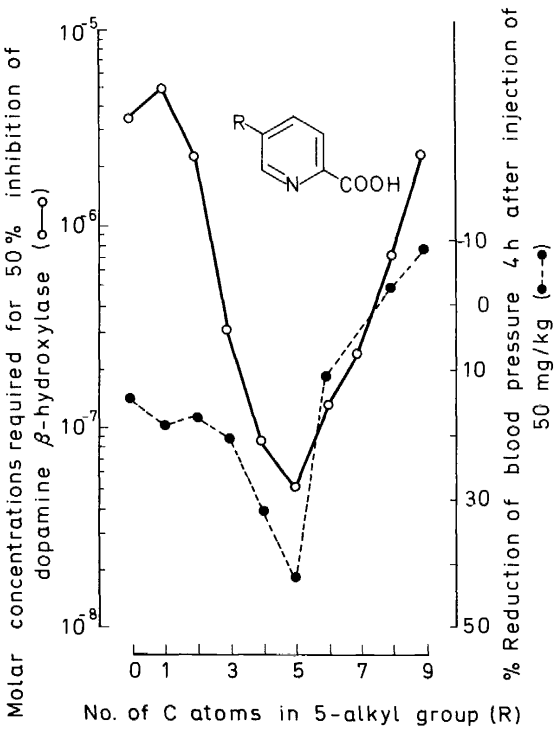


Figure 9.

The metabolism of fusaric acid has been studied with the collaboration of Takemoto, Kubota and Matsuzaki, of the Research Laboratories of Banyu Co. We have not yet isolated all the metabolites of fusaric acid; however, those shown in *Figure 10* have been isolated from the urine of patients who had received fusaric acid. In general, most of the fusaric acid

orally administered is metabolized and less than five per cent appeared in the urine unchanged. All metabolites containing the picolinic acid moiety have about one tenth or less than one tenth the activity of fusaric acid to inhibit dopamine β-hydroxylase (*Figure 10*). The information on the metabolism of fusaric acid in animals and humans can be used in the development of new and effective fusaric acid-related compounds.

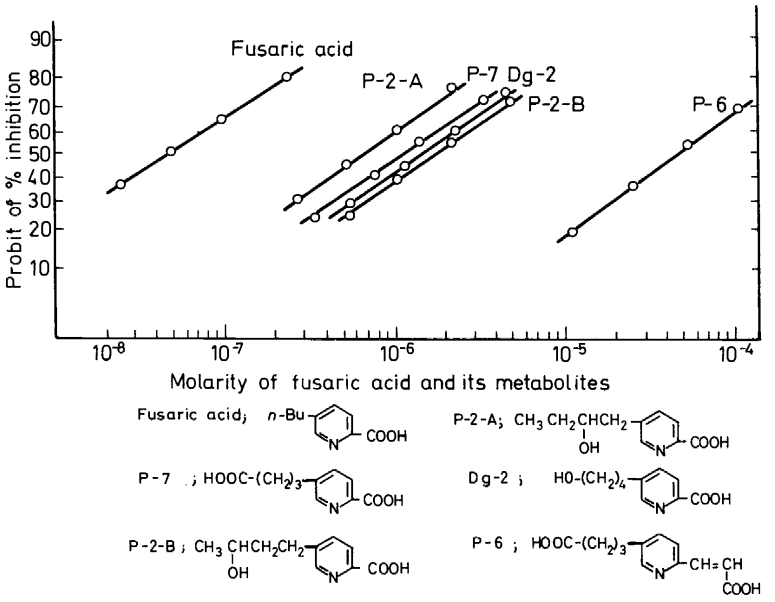


Figure 10.

In our study of dopamine β-hydroxylase inhibitors, we found an effective agent produced by bacteria which had grown on the mushroom and classified as *Pseudomonas*. The active agent was found to be a new compound and named dopastin.

It shows strong laevoratation, $[\alpha]_D^{20} = -250°$ (0.5% ethanol), and is acidic with a pK_a' of 5.2. The formula, $C_9H_{17}N_3O_3$, was calculated from the results of the elemental analysis and its titration. All protons of this molecular formula were identified by n.m.r. in deuterobenzene. The presence of a crotonyl moiety and a 2-substituted isopentyl amino moiety was shown by n.m.r. through application of the double resonance technique. Hydrolysis in 1N hydrochloric acid at 105° for three hours gave crotonic acid which was determined by gas chromatography of its methyl ester. Dopastin is positive in the Rydon–Smith reaction indicating the presence of the peptide bond and positive in the Lieberman reaction suggesting the presence of a nitroso moiety. This information suggested the structure shown in *Figure 11* and this structure was further supported by high resolution mass spectroscopy which indicated the fragments: $C_9H_{17}N_2O_2$ (calc. 185.129; found

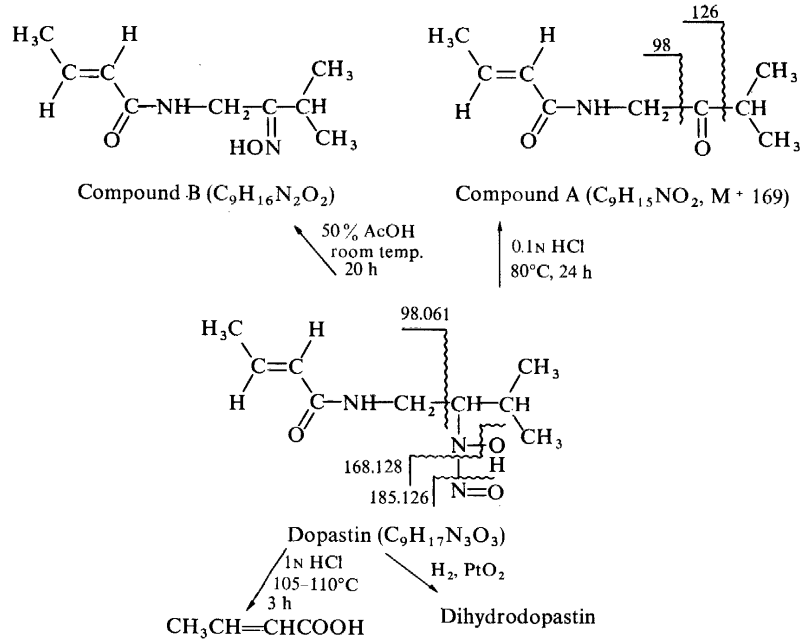


Figure 11.

185.126); $C_9H_{16}N_2O$ (calc. 168.126; found 168.128); C_5H_8NO (calc. 98.061; found 98.061). The structure was confirmed by degradation studies. Mild hydrolysis of dopastin in 0.1N hydrochloric acid at 80° for 24 hours gave the compound A, shown in *Figure 11.* The molecular formula $C_9H_{15}NO_2$ was shown by mass spectroscopy. The crotonyl moiety and the isopropyl moiety were shown in compound A by n.m.r. taken in deuterbenzene. The multiplet signal of methylene adjacent to the amino in the 2-substituted isopentyl amino moiety in dopastin collapsed to the doublet in compound A with the lower chemical shift. Compound A was negative in the Lieberman reaction but positive in the 2,4-dinitrophenylhydrazine reaction indicating the presence of carbonyl. Thus, *N*-(2-oxoisopentyl)crotonamide was proposed as the structure of compound A. This structure of compound A was also supported by mass spectroscopy, as shown in *Figure 11.* Hydrolysis of dopastin in 50 per cent acetic acid at room temperature for 20 hours gave compound B, $C_9H_{16}N_2O_2$. The n.m.r. of compound B was similar to that of compound A except for a singlet at δ 9.7 which could be assigned to an oxime proton. Hydrogenation of dopastin on platinum oxide gave dihydrodopastin.

Dihydrodopastin and dopastin are equally active in inhibiting dopamine β-hydroxylase. Compound A and compound B are both inactive. Therefore, a nitrosohydroxylamino moiety is thought to be essential for activity. Among known compounds, an antitumour antibiotic, alanosine produced

by *Streptomyces alanosinicus*[30] and fragin produced by *Pseudomonas* contain the nitrosohydroxylamino moiety. Alanosine is L-2-amino-3-nitrosohydroxylaminopropionic acid and fragin[31, 32] is *N*-(2-nitroso-hydroxylaminoisopentyl)capryl amide. We found that alanosine is 50 times less active in inhibiting dopamine β-hydroxylase than dopastin, and is very toxic. Dopastin and dihydrodopastin inhibiting dopamine β-hydroxylase also show the hypotensive effect. It is interesting that fusaric acid and dopastin inhibiting dopamine β-hydroxylase have a low toxicity in animals except for lowering of blood pressure, but they exhibit phytotoxicity in inhibiting the germination of plant seeds.

The finding of inhibitors of tyrosine hydroxylase and dopamine β-hydroxylase in culture filtrates of microorganisms stimulated us to extend our study to inhibitors of other enzymes. We are getting successful results in isolating inhibitors of catechol *O*-methyltransferase, histidine decarboxylase etc. In our screening of an inhibitor of monoamine oxidase, an active agent found in a culture filtrate of a streptomyces was phenethylamine, a known inhibitor which had been synthesized. An inhibitor of rat liver xanthine oxidase and milk xanthine oxidase was obtained from actinomycetes and was found to be 5-formyluracil. It inhibits the oxidation of xanthine to uric acid by these xanthine oxidases. This inhibitor is oxidized by these enzymes to 5-carboxy-uracil. 5-Formyluracil causes renal toxicity through accumulation of xanthine in the kidney.

As described above, most enzyme inhibitors obtained from culture filtrates of microorganisms have structures which cannot be predicted from the structures of substrates and cofactors. The argininal moiety in leupeptin and antipain and 4-amino-3-hydroxy-6-methylheptanoic acid moiety in pepstatin cannot be predicted as the active groups for inhibition from the structures of the substrates of proteases. Oudenone is competitive with 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, a cofactor of tyrosine hydroxylase, and is not competitive with tyrosine. Fusaric acid and dopastin are competitive with ascorbic acid, a cofactor of dopamine β-hydroxylase, and are not competitive with dopamine. However, there are no structural similarities between these cofactors and these inhibitors. It can be said that at present we have insufficient knowledge of enzyme reactions to predict the structures of specific inhibitors. Our studies during the last six years have indicated that enzyme inhibitors produced by microorganisms are a promising new and fruitful area, helping us to find new biologically active natural products, which hopefully will lead to useful drugs. It can be said that the investigation of specific enzyme inhibitors produced by micro-organisms is a valuable new field of application for modern organic chemistry.

REFERENCES

[1] B. Kassell, *Methods in Enzymology*, **19**, 839 (1970).
[2] S. Rakhit, *Canad. J. Biochem.* **49**, 1012 (1971).
[3] I. S. Bhatia and S. Sudershan, *Experientia*, **26**, 1078 (1970).
[4] D. S. Duch and N. Kirshner, *Biochim. Biophys. Acta*, **236**, 628 (1971).
[5] T. Aoyagi, T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda and H. Umezawa, *J. Antibiotics*, **22**, 283 (1969).

[6] S. Kondo, K. Kawamura, J. Iwanaga, M. Hamada, T. Aoyagi, K. Maeda, T. Takeuchi and H. Umezawa, *Chem. Pharm. Bull.* **17**, 1896 (1969).
[7] K. Maeda, K. Kawamura, S. Kondo, T. Aoyagi, T. Takeuchi and H. Umezawa, *J. Antibiotics*, **24**, 402 (1971).
[8] K. Kawamura, S. Kondo, K. Maeda and H. Umezawa, *Chem. Pharm. Bull.* **17**, 1902 (1969).
[9] B. Shimizu, A. Ito, H. Saeki, A. Saito, E. Oki, K. Maeda and H. Umezawa. *Abstracts*, p. 32, of the 15th Symposium on the Chemistry of Natural Products at Nagoya, Japan, on 18 October 1971.
[10] T. Aoyagi, S. Miyata, M. Nanbo, F. Kojima, M. Matsuzaki, M. Ishizuka, T. Takeuchi and H. Umezawa, *J. Antibiotics*, **22**, 558 (1969).
[11] H. Umezawa, T. Aoyagi, H. Morishima, S. Kunimoto, M. Matsuzaki, M. Hamada and T. Takeuchi, *J. Antibiotics*, **23**, 425 (1970).
[12] H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada and T. Takeuchi, *J. Antibiotics*, **23**, 259 (1970).
[13] H. Morishima, T. Takita, T. Aoyagi, T. Takeuchi and H. Umezawa, *J. Antibiotics*, **23**, 263 (1970).
[14] M. Fukumura, S. Satoi, N. Kuwana and S. Murao, *Agric. Biol. Chem.* **35**, 1310 (1971).
[15] T. Aoyagi, S. Kunimoto, H. Morishima, T. Takeuchi and H. Umezawa, *J. Antibiotics*, **24**, 687 (1971).
[16] T. Aoyagi, M. Yagisawa, M. Kumagai, M. Hamada, Y. Okami, T. Takeuchi and H. Umezawa, *J. Antibiotics*, **24**, 860 (1971).
[17] M. Kumagai, Y. Suhara, T. Aoyagi and H. Umezawa, *J. Antibiotics*, **24**, 870 (1971).
[18] H. Umezawa, T. Takeuchi, H. Iinuma, K. Suzuki, M. Ito and M. Matsuzaki, *J. Antibiotics*, **23**, 514 (1970).
[19] M. Ohno, M. Okamoto, N. Kawabe, H. Umezawa, T. Takeuchi, H. Iinuma and S. Takahashi, *J. Amer. Chem. Soc.* **93**, 1285 (1971).
[20] S. Ayukawa, T. Takeuchi, M. Sezaki, T. Hara and H. Umezawa, *J. Antibiotics*, **21**, 350 (1968).
[21] S. Ayukawa, M. Hamada, K. Kojiri, T. Takeuchi, T. Hara, T. Nagatsu and H. Umezawa, *J. Antibiotics*, **22**, 303 (1969).
[22] M. Sezaki, S. Kondo, K. Maeda, H. Umezawa and M. Ohno, *Tetrahedron*, **26**, 5171 (1970).
[23] R. J. Taylor, C. S. Stubbs and L. Ellenbogen, *Biochem. Pharmacol.* **19**, 1737 (1970).
[24] I. Yamamoto, K. Nitta and Y. Yamamoto, *Agric. Biol. Chem.* **26**, 486 (1962).
[25] M. Uemura and T. Sakan, *Chem. Commun.* 921 (1971).
[26] H. Hidaka, T. Nagatsu, T. Takeya, T. Takeuchi, H. Suda, K. Kojiri, M. Matsuzaki and H. Umezawa, *J. Antibiotics*, **22**, 228 (1969).
[27] T. Yabuta, K. Kambe and H. Takeshi, *J. Agric. Chem. Soc. Japan*, **10**, 1059 (1934) in Japanese.
[28] H. Suda, T. Takeuchi, T. Nagatsu, M. Matsuzaki, I. Matsumoto and H. Umezawa, *Chem. Pharm. Bull.* **17**, 2377 (1969).
[29] M. Ogata, *Igaku no Ayumi*, **77**, 534 (1971) in Japanese.
[30] C. C. Lancini, A. Diena and E. Lezzari, *Tetrahedron Letters*, 1769 (1966).
[31] A. Murayama and S. Tamura, *Agric. Biol. Chem.* **34**, 122 (1970).
[32] A. Murayama and S. Tamura, *Agric. Biol. Chem.* **34**, 130 (1970).