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—Review—

Industrial Application of a Macrotetrolide Antibiotics

Kunio Ando and Yoshiharu Nawata

Research Laboratories
Chugai Pharmaceutical Co., Ltd.
Takata, Toshima
Tokyo 171, Japan

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Tetranactin is, so far as we know the first pesticidal antibiotic ever commercialized. It has been in use in Japan as an agricultural miticide since 1971.

Environmental pollution is now a serious problem all over the world. Especially chemicals such as pesticides sprayed over wide areas are apt to cause ecological changes and subsequent destruction of environment. Because of the dense population in Japan, environmental pollution is a particulariy severe problem. Much of Japan is mountainous and the area of cultivated land is relatively small. (Among some of the more densely populated countries of the world (Fig. 1), Japan ranks fourth, after Korea. Holland and Belgium, in density relative to the countries' total area. But it ranks first in density relative to cultivated area.) Japanese farmers have developed intensive agriculture because they have to harvest big crops from limited land. This high productivity is supported by abundant use of commercial fertilizers and pesticides. Unfortuntely, excessive use of chemicals has brought about environmental pollution to our country.

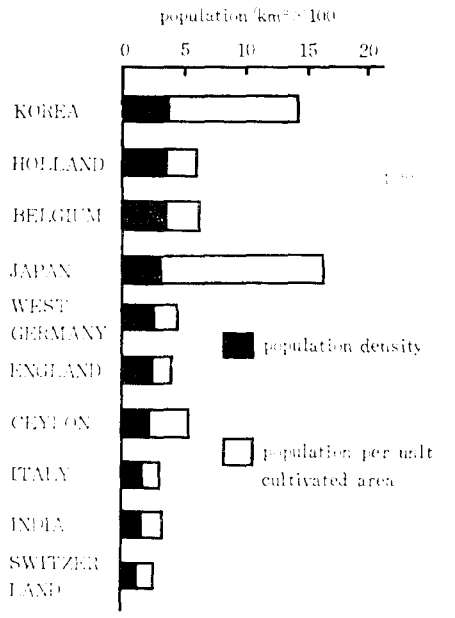

Fig. 1. Population Densities.[1]

There are two alternative ways to avoid pollution. One is the legal prohibition of the use of agricultural chemicals. The other is research and development of less detrimental chemicals to replace the destructive ones. The latter approach is the more practical one.

The ideally harmless pesticide should have a number of properties, among which we think two are most important: selective toxicity and ready decomposability into small. safe molecules such as H_2O, CO_2 and NH_3. Antibiotics have such ideal properties. They are produced by microorganisms and such orqanic products are readily decomposable by other microorgansims, and even by the producing ones themselves. For this reason research and development of agricultural antibiotics have been most actively and extensively prsued in Japan.

Some of these antibiotic pesticides are (Fig. 2): blasticidin s and kasugamycin, used in rice blast disease: validamycin, developed for rice sheath blight: and polyoxins control fungal infection on

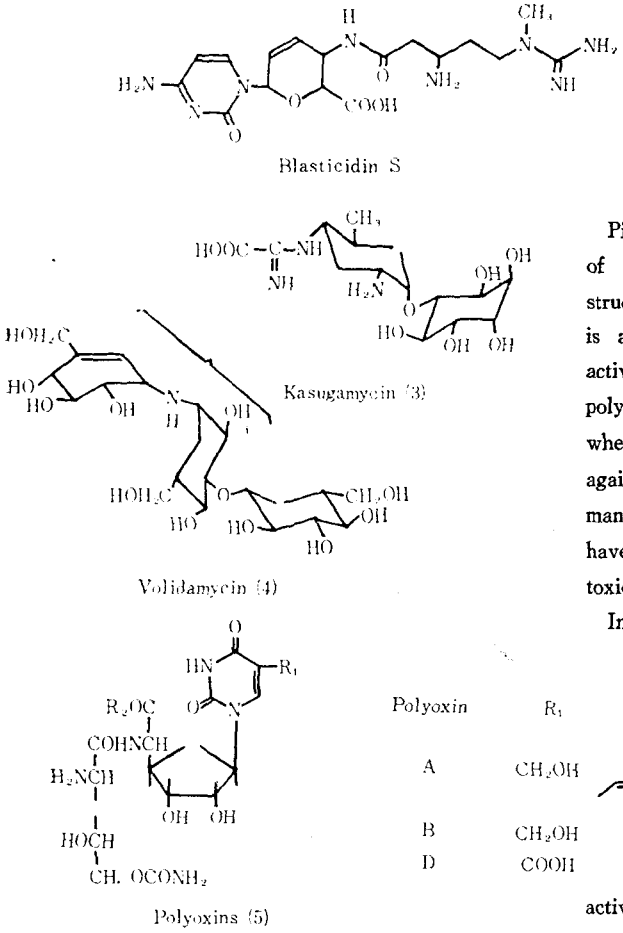

Fig. 2. Agricultural Antibiotics for Fungal Infections

fruits and vegetables. These are contributing greatly to agricultural productivity in Japan.

Although much has been done to develop fungicides in Japan not much work has been done on the pesticides. Because of this the Chugai Laboratories have paid particular attention to the pesticidal antibiotics. More than a decade ago we started work on them. Figure 3 shows typical examples of these entities which have been developed in our screening program.

Piericidin was first isolated by Takahashi et al. of Tokyo University. It is interesting that the structure is similar to that of ubiquinones. Aureothin is an antibiotic with a nitro group which is active against certain kinds of insects and mites. polyether antibiotics, such as monensin kill insects when topically applied. Antimycins are also active against certain kinds of mites. Besides these, many other antibiotics with pesticidal properties have been reported, but most ofthem are highly toxic to warm-blooded animals.

In our screening we used the Azuki bean weevil as a test insect and isolated the new antibiotic, tetranactin, from Streptomyces culture.[10] The Azuki bean weevil is a tiny insect which we can easily propagate with the Azuki bean. When isolated the active principle tetranactin froms a large prism crystal. This compound is of particular value as a pesticide because it has very low toxicity for non-target organisms, especially warm-blooded animals, and readily decomposes into carbon, oxygen and hydrogen metabolites[11].

We studied the active principle chemically and

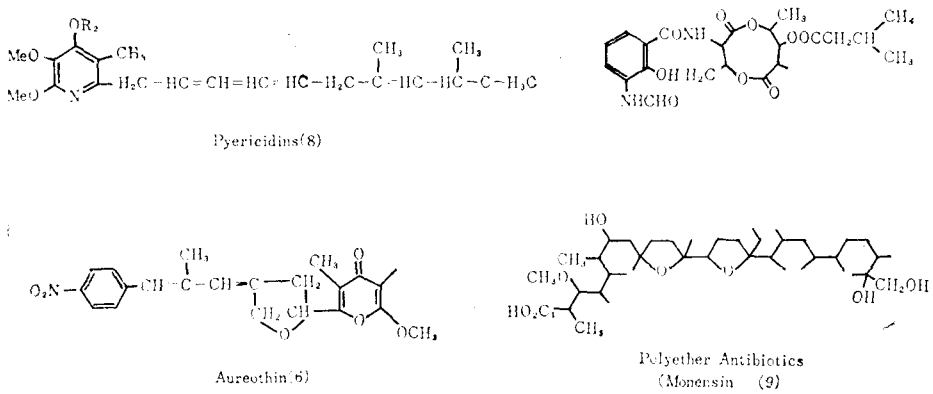

Fig. 3. Some Pesticidal Antibiotics Developed in the Chugai Laboratories.

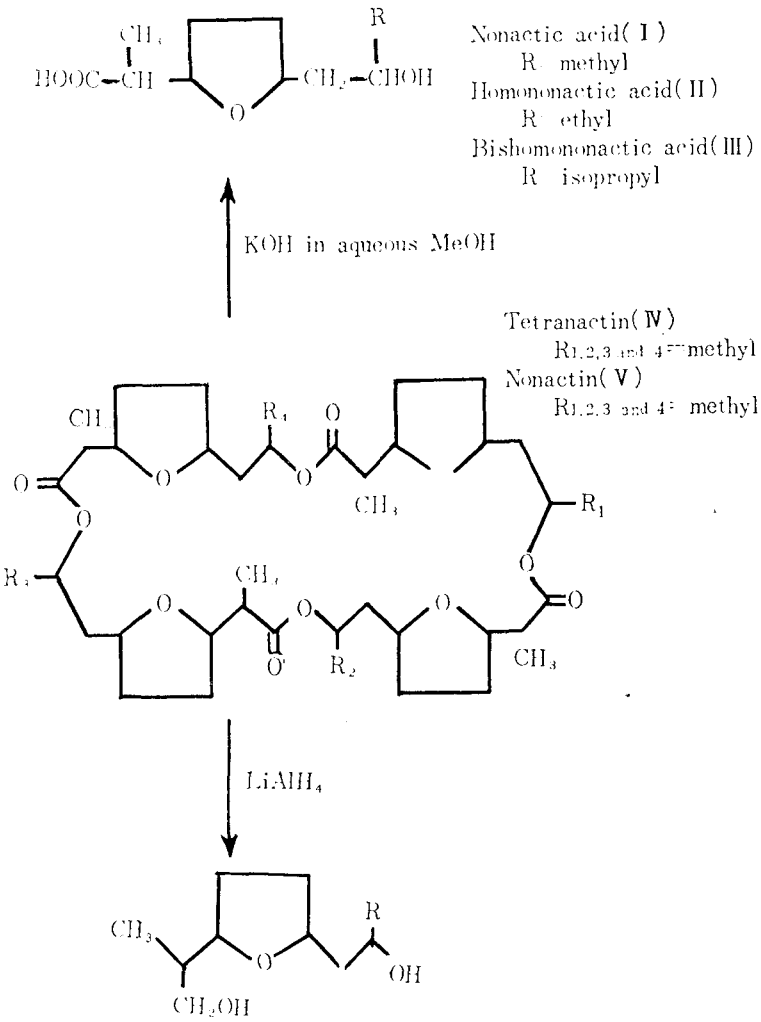

Fig. 4. Tetranactin and Its Chemical Derivatives.

found it belongs to the macrotetrolide class of antibiotics. We named it tetranactin. Its structure is shown in Figure 4. It is composed of four equal structural units called homonactic acid. On alkaline hydrolysis, we obtained homononactic acid as a methyl ester. On reduction with $LiAlh_4$ we isolated diol with a tetrahydrofurane ring as a sole product. In this way we could reveal the structure as shown in Figure 4. [12.17].

Homononactic acid has four asymetric carbons in its molecule. Therefore the antibiotic possesses 2^{16} stereoisomeres. We elucidated the stereochemical structure by x-ray crystaography. Figure 5 shows the computer drawn structure of tetranactin. The large balls represent oxygens and carbons, the small balls hydrogen. The molecule is rather fiat and twisted.

The macrotetrollde antibiotic is called an ionophre antibiotc because it specifically forms a com-

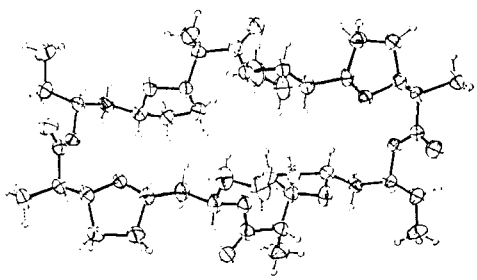

Fig. 5. A computer-drawn Represention of Tetranactin.

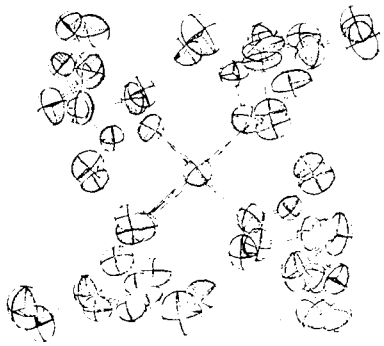

Fig. 6. Crystalline Structure of the Tetranactin-rubidium Complex.

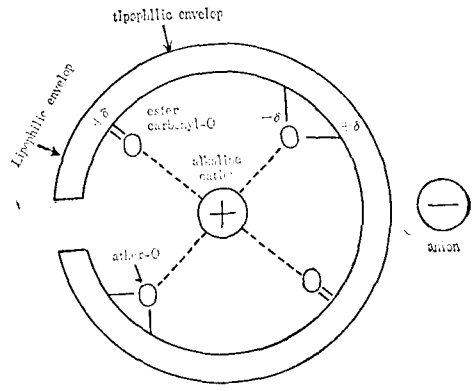

Fig. 7. Diagram of the Way Tetranactin Works.

Table 1. Pesticidal activity of tetranactin.

Insect or mite tested	Method	LC_{50} or LD_{50}
Tetranychus cinnabarinus adult	spray	4.8 μg/ml
Aleuroglyphus ovatvs adult	film contact	238.8 μg/cm²
Tyrophagus dimidiatus adult	film contact	80.0 μg/cm²
Callosobrucuhus chincesis adult ♁	topical appl.	0.8 μg/weevil
Muscc domestica adult ♁	topical appl.	>4.0 μg/fly
Blattclla germanica adulet ♂	topical appl.	>4.0 μg/cockroach
Culex piriens molestus larva	dipping	7.0 μg/ml
Chilo suppressalis larva	topical appl.	>4.0 μg/larva
Prodenia lilura larva	topical appl.	>4.0 μg/larva
Myzus persicae adult	spray	340 μg/ml
Pseudococcus comcomstocki adult	film contact	>1.0 mg/cm²
Ncphottetix cincticeps adult	film contact	>400 μg/ml

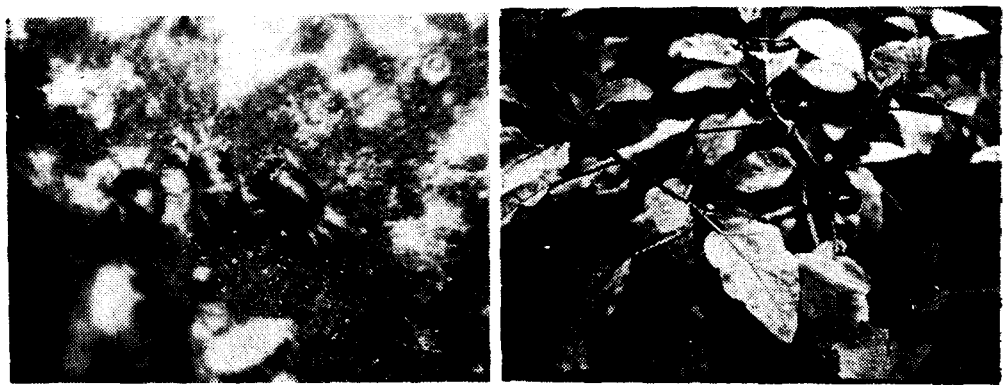

Fig. 8. Carmnie Spider Mites on Eggplant.

plex with alkaline cations. Figure 6 depicts the crystalline structure of tetranactin-rubidium complex. The molecule is globular in contrast with the free tetranactin. The rubidium ion is located at the center of the globule and is fixed with an ion-dipole of ether and carbonyl oxygens. The surface of the globule is covered with lipophilic moieties such as ethyls. methyls and methylenes. Therefore the complx readily dissolves in most organic solvents such as chloroform, dichloromethane and ethylacetate. [15.16]

This how the the complex works. If an alkaline cation apprpaches the free tetranactin, the antibiotic envelopes the ion. (Fig. 7) Both the ether oxygen of tetrahydrofurane and the ester carbonyl oxygens are slighty charged electronegatively due to ion dipole moment, so that they interact with the cation to fix it at the center of the globule. The globule is lipophilic due to its outer covering of lipophilic moieties, so the antibiotic makes the cation lipophilic.

Specificity of activity is an outstanding property of tetranactin (Table I). It exerts potent pesticidal activity against the carmine spider mite (*Tetranychus cinnabarinus*) alone. The LC_{50} is 4.8 $\mu g/ml$ with the spray method. The Azuki bean weevil and the larva of the mosquito (*Culex pipiens molestus*) are moderately susceptible to the antibiotic. Other pests, for example the house fly and cockroach are insensitive to it, Among pests the mite is particularly troublesome because it readily acquires resistance to pesticides due to quick regeneration time and vigorous eeproductivity. Therefore we decided to develop tetranaction as a miticide.

The carmine spider mite (*Tetranychus cinnabarinus*) (Fig. 8) is a minute animal with eight legs. When it grows on the leaves of eggplants the plants wilt and the harvest is severely damaged.

The LC_{50}'s for four species of mites are shown in Table II. Concentrations of less than 10 $\mu g/ml$ are lethal to the adults of the carmine spider, two-spotted spider. kanzawa spider and European red mites. However. tetranactin is only weakly ovocidal against these mites.

We corroborated the miticidal activity in field trials by spraying tetranactin suspensions on apple

Table. 2. Miticidal Activity of tetranactin.

Mites tested	Miticidal activity (LC_{50}, ppm)	
	Adule	Egg
T. cinabarinus	4.8	59
T. urticae	9.4	80
T. kanzawa	7.2	110
P. ulmi	2.2	50

Spray mcthod was uscd.

Table. 3. Miticidal Activity of Tetranactin in Field Tests.

Agants	Mites	Concn (ppm)	No. of mites[a] before spraying	Population of mites at indicated days (%)[b]				
				6	10	16	24	32
Tetranactin	*P. ulmi*	133	511	1.8	1.0	1.2	0.6	0.6
		100	493	2.4	11.6	1.6	.8	1.6
	T. kanzawa	133	780	1.4	7.6	2.3	5.5	2.3
		100	430	3.5	6.9	9.3	9.3	.0
Dicofol	*P. ulmi*	200	230	0.0	5.0	6.7	5.0	5.0
	T. kanzawa	200	208	2.4	3.4	11.5	21.6	11.5
Control	*P. ulmi*	—	240	408.2	232.1	153.1	130.0	88.9
	T. kanzawa	—	114	606.1	362.3	245.6	42.1	24.6

[a] Numbers of mites/20 leaves collected randomly.

[b] Mite populations were cxpresscd as follows: no. mites after spraying/no. mites before spraying×100 (%).

Table 4. LD_{50}'s for Warm-Blooded Animals.

Species	Route	Acute toxicity (LD_{50}, mg/kg)
Mouse	po.	> 25000
Mouse	ip.	> 500
Rat	po.	> 2500
guinea pig	po.	> 2000
Quail	po.	> 2000
Rabbit	po.	> 2000

trees on whose leaves kanzawa spider mites and Evropean red mites are naturally parasitic. For comparison we sprayed dicofor. another commonly used miticide, on adjacent plots. As the last column of Table III shows, on the 32 nd day after spraying the mits populations were reduced to 2.3 % or less of their prespraying numbers where tetra nactin was used. Dicofor's effectiveness was appreciably less.

Another of tetranactin's favorable characteristics is its safety for warm-bloodod animals (Table IV). LD_{50}'s for these animals are so high as to be virtually incalculable. Mice, for example, can tolerate a single oral dose of 25 g/kg. we made a two-year study of chronic toxicity in rats and dogs. They received more than 100 mg/kg every day for two years and remained normal. This low toxicity is partly partly due to poor assimilation. We prepared ^{14}C-labelled tetranaction by formen-

Table 5. Tissue Distribution and Urinary and Fecal Excretion of ^{14}C-Labelled Polynactin Complex in Mice.

	% of dose		
	1 hr mean ± SE	24 hr mean ± SE	72 hr mean ± SE
Blood	0.00±0.00	0.01±0.01	0.00±0.00
Liver	0.02±0.01	0.04±0.02	0.01±0.00
Kidney	0.00±0.00	0.00±0.00	0.00+0.00
Carcass	0.50±0.66	0.47±0.46	0.00±0.00
Intestine	92.38±7.70	37.82±15.66	0.00+0.00
Urine	0.04±0.01	0.26±0.06	0.75±0.32
Feces	0.00±0.00	55.19±15.26	84.67±2.75
Total	92.94±7.68	93.79±7.08	85.42±2.57

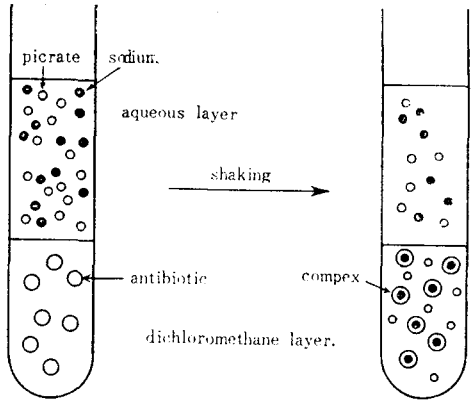

Fig. 8. Principle of the Assay Method.

tation and administered it orally to mice (Table V). The antibiotic is so little absorbed through the intestines that hardly any of it is found in the other organs. Almost all of the radioactiiity (99.1 %) was recovered from the feces within 72 hours after injection. [19]

We developed a method for the quantitative determination of tetranctin by utilizing its complex forming propery. [21,22] Figurre 8 shows the principle of the assay method. The antibiotic and its complex with sodium picrate are readily soluble in dichloromethane but insoluble in water. Sodium picrate is soluble in water but insoluble in dichloromethane. If the antibiotic is present in the lower dichloromethane layer, vigorous shaking with aqueous sodium picrate generates tetranactin-sodium

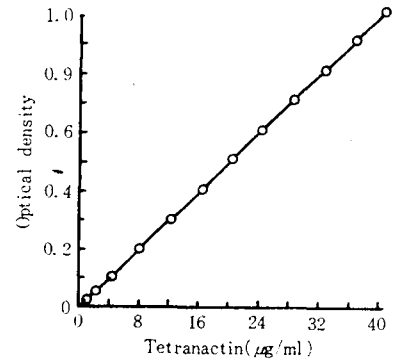

Fig. 9. Standard Curve for Tetranaction Method: see in the text

complex. The sodium ion is extracted in association with the picrate ion owing to the interaction between them. Therefore, we can estimate the antibiotic by measuring absorption of the transferred picrite ion. This assay method gives a straight calibration curve from O to 40 $\mu g/ml$ (Fig. 9). The method is simple. reproducible and accurate. Recently Ishibashi and his colleagues at Kyushu University reported[23] a more sensitive assay method. They used a fiuorescent compound 8-aniline-1-naphthalene-sulfonate instead of picrate. Their method is approximately 10~100 times more sensitive than ours.

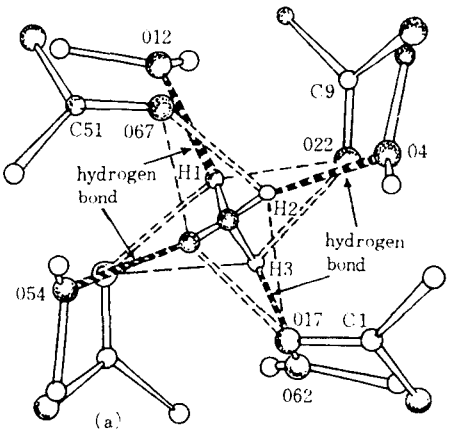

Fig. 10. Structure of the Tetranactin-Ammonium Thiocyanide Complex.

(Nawata elucidated the crystalline structure of tetranactin-ammonium thiocyanide complex[24]. The ammonium complex (Fig. 10) is similar in gross shape to the alkaline cation complexes. However. in the latter the alkaline cations interact with both ether and carbonyl oxygen, whereas the ammonium ion forms hydrogen bonds with the ether oxygen located tetranedrally. [25,26] Nawata anticipated that the ammonium ion would be able to form a more selective complex than alkaline cations due to the hydrogen bonds. In fact, Ishibashi and his colleagues[27] demonstrated that the ammonium ion can bind more firmly with tetranactin.)

(Hayano and Shinozuka of Tokyo University applied this property using the ion-selective electrode for ammonium determination[28] (Fig. 11). A than film, composed of polyvinyl-chloride, plastisizor and tetranactin is fixed at the bottom of the antibiotic electrode. Voltage difference between the two electrodes is detected by the ion meter and recorded.

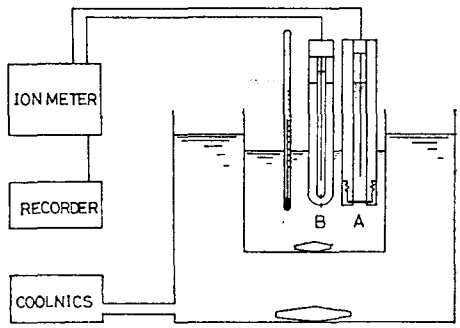

Fig. 11. Ion-selective Electrode for Ammonium Determination.

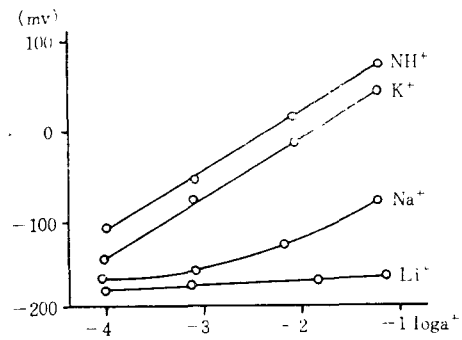

Fig. 12. Ion Selectivity of the Electrode.

The ion selectivity of this electrode is demonstrated in Figure 12. The lithium ion has no affinity for the antibiotic, so naturally there is no concentration-dependent response. Selectivity of the sodium ion. which is well below that of the ammonium. is one three-hundredths. Both the ammonium and potassium ions have linear concentration-dependent response, but the selectivity of the latter is approximately one-third that of the former. Therefore an appropriate way of separation is necessary for quantitative determination of the ammonium ion. The film is durable and the results are highly reproducible, so we believe this device

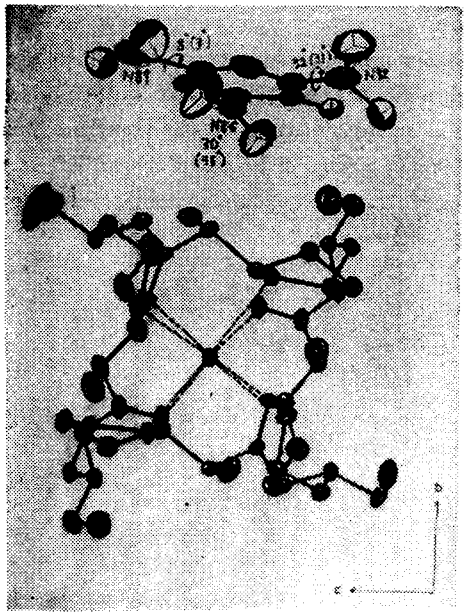

Fig. 13. Tetranactin Rubidium Pipcrate.

is promising for ammonium ion determination.)

(Anraku and his colleagues reported that a liposome membrane containing valinomycin plus an uncoupler, 3. 5-di-tertial-butyl-4-hydroxybenzylidenzylidenemalononitrile. allows potassium inos to pass more readily.[29,30] This result indicates that there are some kinds of interaction between the neutral ion carrier and the uncoupler in the membrane. Nawata selected rubidium picrate as an uncoupler and crystallized tetranactin rubidium picrate complex for x-ray crystallography. He tried to solve the interactions between the neutral ion catrier and the uncoupler in the crystalline state (Fig. 13). The blue balls represent oxygen. the black balls carbon atoms. The orange ball is the rubidium ion. The gross shape of the tetranactin rubidium complex is similar to that of the rubidium thiocyanide complex. However. we can demonstrate clear interactions between picrate and the complex, as shown in Figure 14. Here the green and rose dotted lines indicate possible oxygen and hydrogen interactions.[32] The rose dotted line indicates interactions with the upper complex and the green dotted line shows that with the complex below. The solid orange line is picrate ion. Oxygen of the nitro groups and phenolate located near the aliphatic groups of the complex interact with methyl, methylene and methine. Therefore, though the picrate ion is originally flat, the nitro groups rotate according to the oxygen-hydrogen interactions. Consequently the neqative charge of the picrate ion is not localized at the phenolate oxygen, but diffuses to some parts of the molecule, as shown in Figure 15. The carbon-to-carbon bond length in the picrate ion is longer than that in the ordin the ordinary aromatic compounds. Thus a negative charge diffuses this part cf the molecule. This diffusion of the negative charge occurs in solution. thus reducing the interaction of the phenolate ion with water. Once the complex is formed on the interface between water and organic solvent, the phenolate ion readily transfers into the organic solvent layer due to the loss of the interaction with water.)

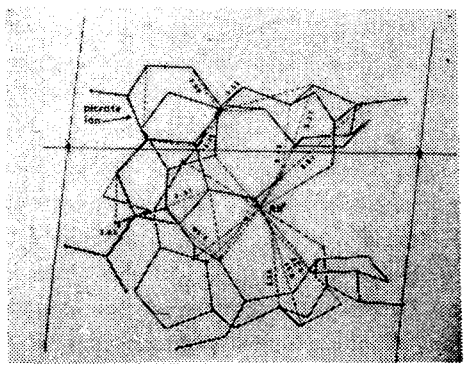

Fig. 14. Interaction Between Tetranactin-rubidium Complex and Picrate ion.

Reganding the mode of action of tetranactin. the macrotetralide antibiotic acts as an uncopler

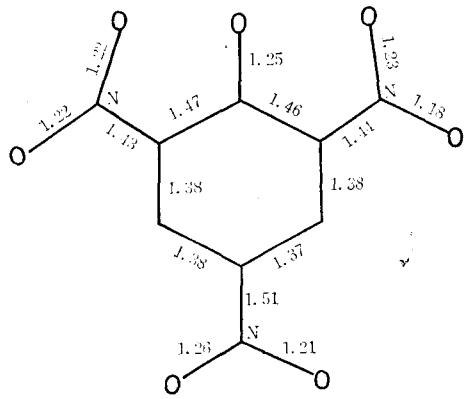

Fig. 15. Bond Iength of Complexed Picrate Ion.

Table. 6. Residual Activity of Tetranactin Day Alm.

Concn (ppm)	Residual miticidal activity after spray with water (% mite kill) on day				
	1	2	4	7	11
	P. ulmi				
200	100	100	100	94.1	86.0
100	100	100	100	40.0	0.0
	T. urlicae				
200	100	100	74.4	25.0	.0
100	100	100	74.5	16.0	.0

in oxydative phosphorylation. We also confirmed that tetranactin causes uncopling in cockroach mitochondria. However, when large amounts of powdered tetranactin is applied to the mites they do not die. For example, mites placed on leaves covered with tetranactin dry film were quite safe under various humidities. [33] But when water was sprayed on these leaves. all of the mites died soon after spraying (Table VI). This reactivation of the antibiotic dry film is atributable to regeneration of the antibtiotic suspension by free water. So water is an essential factor for the miticidal activity.

We can interpret this reactivation on the basis of the complex formation. All living cells maintain a relatively high potassium content. The intracellular potassium ion is surround by water molecules

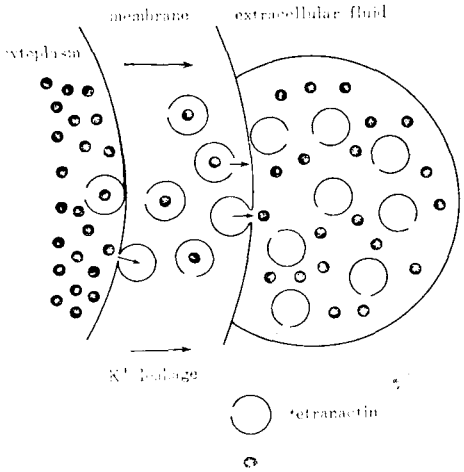

Fig. 15. Potassium Leakage through a Biomembrane Effected by the Antibiotic.

so that it is unable to leak through the biomembrane. If the membrane contains macrotetralide antibiotic, the cation can leak via complex formation (Fig. 15). When the antibiotic aqueous suspension adheres to the body of the mites, the antibiotic (large open circles) is incorporated into the membrane following complex formation with intracellular potassium ions. Once the complex is formed. the cation leaks between inner and outer fluid according to the concentration gradient. That is to say, the antibiotic makes a hole in the membrane which allows the potassium to pass through.

In conclusion, the complex forming property is most important in understanding the biological and chemical properties of tetranactin. We again contrast the difference between the free and the complex. The figure on the right (Fig. 16) is the free molecule. the complex is on the left. Being enveloped with lipophilic moieties, some alkaline

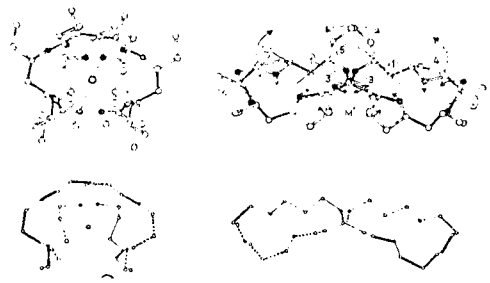

Fig. 16. Gross Structure of the Free (Right) and the Complex (left) Tetranactin.

cations and amines become lipophilic. Moreover, the affinity for these ions varies according to the ion radii and hydrogen bond formation. Ammonium ion has the highest affinity and the affinity of alkaline cations depends upon the ion radii. potassium and rubidum have great affinity. sodium and cesium have moderate affinity. and lithium his none.

One of the great mysteries of nearly all living cells is how they maintain relatively high potassium and low sodium content while surrounded by fluid with a reverse concentration ratio of the two cations. We hope tetranactin may play a role in clarifying this mystery.

Acknowledgement:

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