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Chemistry of photoproteins as interface between bioactive molecules and protein function

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Abstract: Bioluminescence of Okinawan squid includes a photoprotein named *"Symplectin"* which gives light by addition of Na^+ (or K^+). The chromophore is covalently bound-imidazopyrazinone analog to protein. Symplectin was extracted with 0.6M KCl and was analyzed to be composed of 3 different molecular-size proteins. Symplectin A, the smallest photoproteins, was purified by gel permeation chromatography to have 15kd by MALDI/TOF mass spectrometry. The molecular mechanism of this luminescence is described. Bioluminescence is also applied for trace analyses and the chromophore in some new fireflies is chemically analyzed. We made a very sensitive detection system of luciferin to a detection limit of 10^{-16} mol/10μl by using immobilized luciferase and a single photon-counter. An example of the firefly luminescence application is demonstrated in an inhibitory-assay of protein phosphatase activity by inhibitors in the same class of tautomycin/okadaic acid.

Introduction

Many biological phenomena transfer information through organic molecules, in which a number of bioactive molecules play roles in communication among molecules. The molecular-molecular interaction thus stands in an important position for the understanding of these molecular communication. Bioluminescence has peculiar nature to exhibit light as a result of biochemical oxidation; namely a luminescent element-chromophore is recognized by protein and oxidized to emit light. This is itself one of the typical organization of molecular systems but it can also be applied to be a monitoring system of other biochemical reactions. Our interest on molecular mechanism of structure recognition with our organic chemistry as back-ground is summarized in Fig. 1.

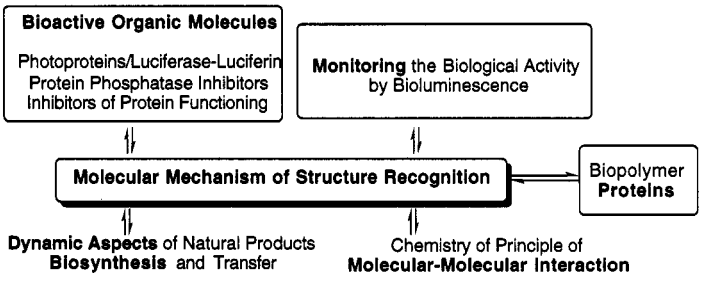

Fig. 1 A photoprotein plays fundamental and applicable roles for bioorganic research.

Symplectin, a photoprotein from Okinawan squid

A photoprotein is a built-in luminous system containing luciferase and luciferin-like chromophore in its molecule, so it waits for the joining of the third triggering element for light emission. We became

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interested in one of these photoproteins from Okinawan squid "Tobi-ika" (Fig. 2), *Symplectotheutis o.*, that emits 460-470 nm blue light from the photogenic organ (Fig. 3).

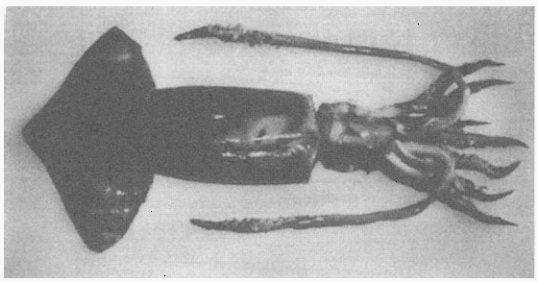


Fig. 2 Luminous Okinawan squid

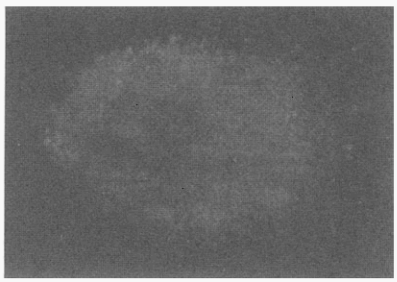


Fig. 3 Luminescence of the photogenic organ

Monovalent metal ions, particularly Na^+ (K^+), and molecular oxygen are the elements necessary to give light besides Symplectin. The chromophore is dehydrocoelenterazine, an oxidative form of a common luminous substance coelenterazine in marine bioluminescence; but it becomes luminescent by conjugate addition of sulfhydryl group of cysteine residue of the protein.[1,2]

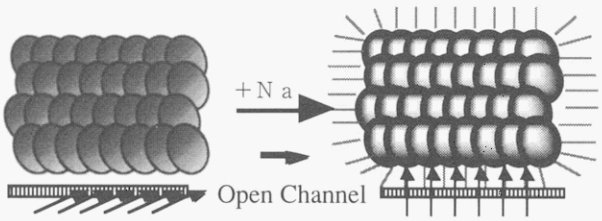


Fig. 4 Photoprotein gives light when Na^+ comes into the photogenic organ through ion channels.

Symplectin is only extractable to water with high concentration of salt, and the salt must be >0.6M KCl to retain the luminescence function. Thus, the homogenized photogenic organs are first washed by <0.4M KCl and then Symplectin (Fig. 5) is extracted in ice-cold 0.6M KCl at pH 5.6, since the luminescence reaction is slow at low temperature (Fig. 6) and at low pH's (Fig 7) under nitrogen atmosphere. Luminescence of a fraction can be monitored by addition of the sample into a buffer of pH 7.8 containing 0.6M KCl at 20°C.

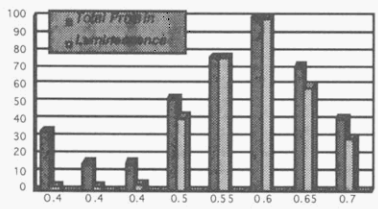


Fig. 5 Extraction of Symplectin (left side bars: total protein).

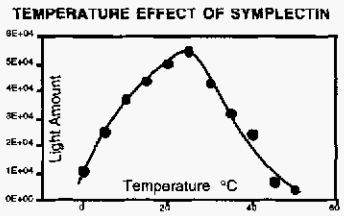


Fig. 6 Effect of temperature on luminescence.

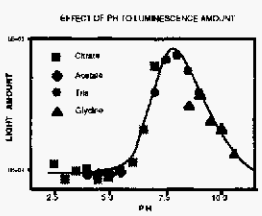
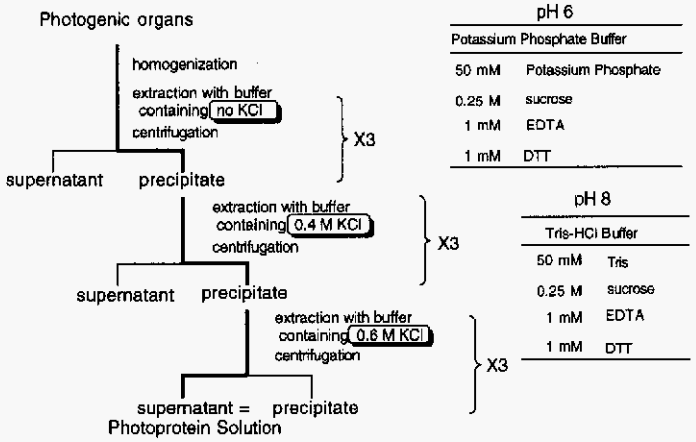


Fig. 7 Effect of pH on luminescence.

We have established the extraction procedure as shown in Scheme 1. Symplectin mainly contains 3 proteins of different molecular sizes (15, 60 and >120 kd; named Symplectin A, B and C, respectively) with luminescence activity. This mixture is separated by a gel-permeation chromatography column with 1M KCl elution. The chromatograms are shown in Fig. 8, detected by uv, fluorescence and luminescence; and the fractions were further analyzed by SDS-PAGE gel electrophoresis. The smallest molecule (Symplectin A) showed its molecular weight of 15,300 Da by MALDI-TOF mass measurement. Amino acid sequence of Symplectin has been analyzed and its further structural studies are in progress. The luminescent mechanism of Symplectin is studied in two aspects: one is biochemical studies and the other one is physical organic studies focusing on what is going on with the chromophore on protein. Differential

fluorescence spectra have supported the mechanism in Scheme 2, but the dark reaction has been supported only from a late-increase of 420 nm fluorescence due to the amino-pyrazine. Some more evidence is being added by means of mass spectrometric method of the whole photoprotein molecule.



Scheme 1 Extraction method of Symplectin.

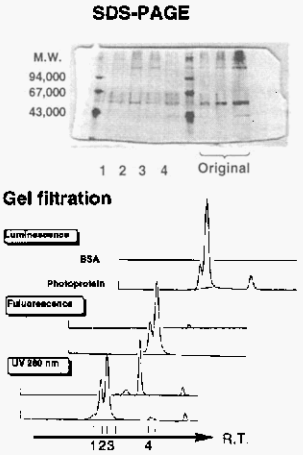
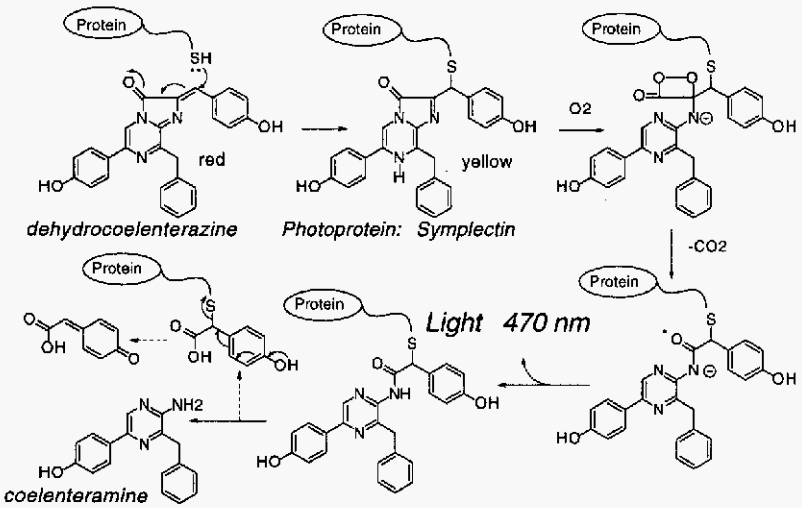


Fig. 8 Isolation of Symplectin A.

The mechanism of luminescence can be examined particularly on the dioxetanone being an extremely short life-time species as a direct precursor of luminescence. We have already reported the evidence of synthetic dioxetanone structure (Fig. 9) by photooxygenation at low temperatures around -78°C in trifluroethanol-methanol as solvent and by the ^{13}C NMR and IR spectra.[4,5,6]



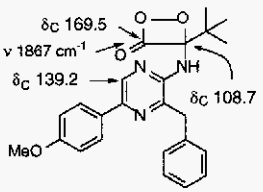


Fig. 9 Luminous dioxetanone.

Model compounds with 100% ^{13}C enriched at either the C2, 3 or 5 position were synthesized by photooxidation. The nmr and ir were taken at -78°C to prove the structure as above.

Scheme 2 Luminescence mechanism of Symplectin.

We have further improved the low-temperature experiments in a mixture of hexafluoroisopropanol-methanol (7:3) to give a higher (40%) yield of the dioxetanone by NMR as shown in Fig. 10. These experiments reconfirmed the structures of peroxide intermediates as shown in Fig. 11. These two peroxides exhibited different properties in thermal stability in different pH of the media as summarized in Fig. 12. Each top-bottom pair in Fig. 12 exhibits the relative luminescence intensity in the two different solvents, while the left-column figures are in slightly acidic (pH 5.6) in DGM (dyglyme solvent), the central-column ones in neutral and the right-column ones in basic. The solid lines constantly showing peaks at -30°C (independent of pH) correspond to dioxetanone. On the other hand, the dotted lines showing different maxima in different pH's in DGM correspond to a dissociation equilibrium of the 2-

hydroperoxide into its anions to attack the lactam carbonyl group and eventually to give light at 470 nm as anion species of the amide.

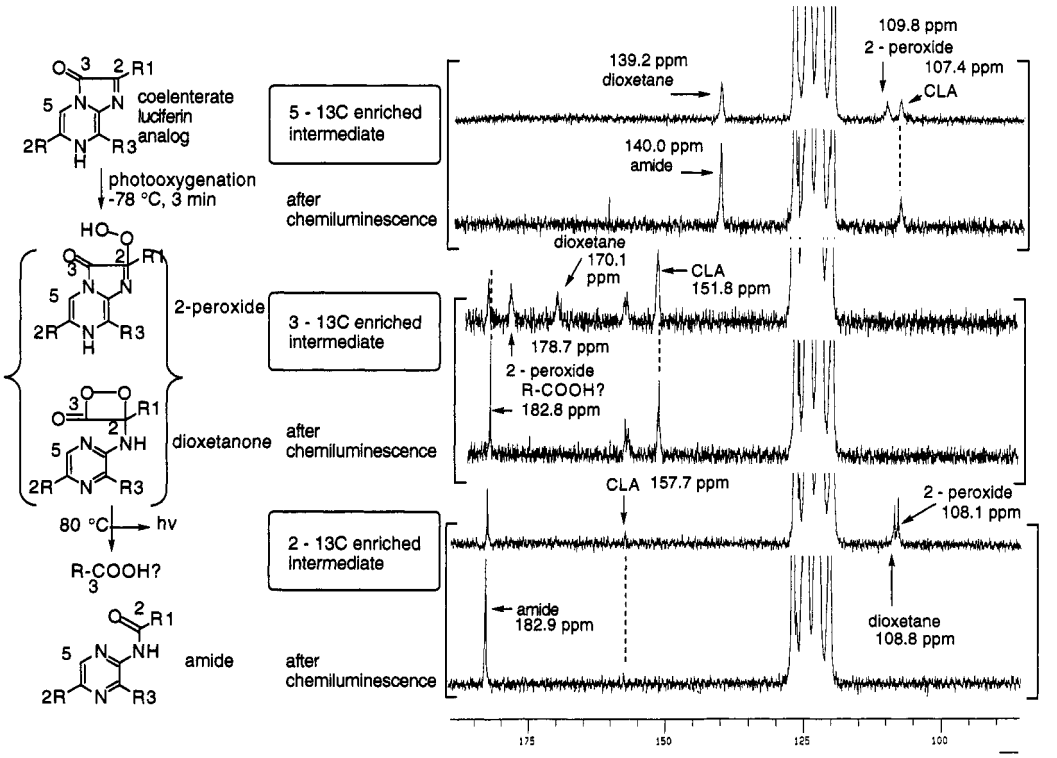


Fig. 10 Photooxygenation of the ^{13}C-enriched analog in $(CF_3)_2CDOD$-CD_3OD (7:3).

Importance lies in the fact that 2-hydroperoxide emits the maximum light at lower temperatures even in acidic or basic media than in neutral medium. This pH dependency suggests different mechanisms for the formation of dioxetanone in different pH's; and thus difference in anionic or neutral amide species. Dioxetanone itself gives 420 nm as a neutral species in acidic or neutral media, but anionic (470 nm) in strongly alkali media.

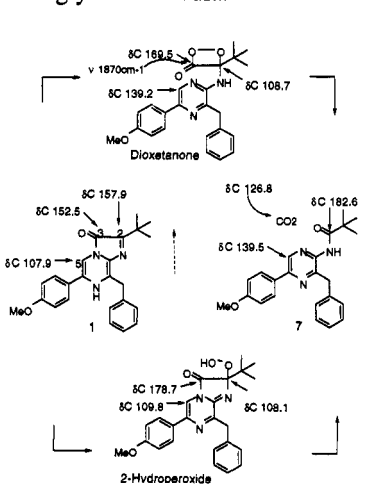


Fig. 11 Assignment of low temperature ^{13}C NMR with 100%-enriched substrate.

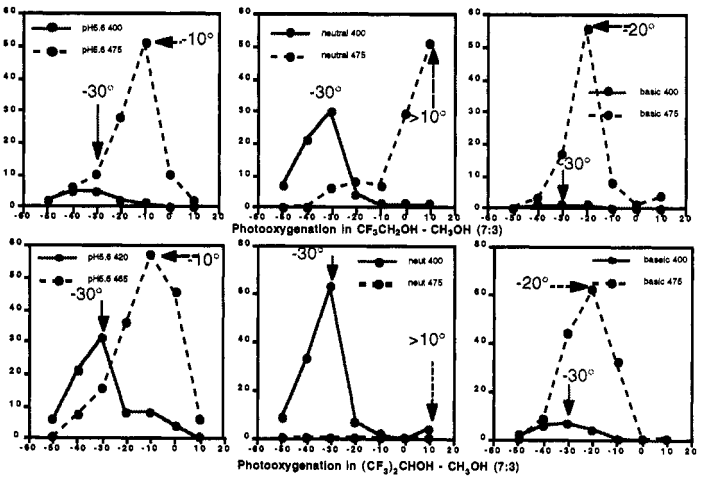


Fig. 12 Effects of pH and temperature to luminescence of 2 different peroxides.

Photoprotein Symplectin may connect with the chromophore through thioether linkage, which is in contrast to the case in aequorin, another photoprotein in jellyfish.

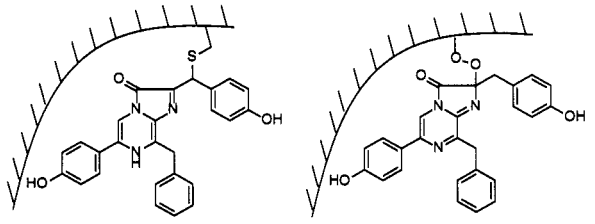


Fig. 13 Photoproteins, Symplectin (Okinawan squid)-the left side and Aequorin (jellyfish)-the right side.

Firefly Luminescence in Malaysia

Firefly luminescence is the most common bioluminescence to people in various places in the world. We have been interested in the different wavelength of different firefly luminescence with maxima between 550-575 nm. The luciferin structure is identical, irrespectively the different luminescence maxima. In Malaysia, Selangor, *Pteroptyx tener* is of abundance in mangrove area and its local name is Kelip-Kelip. All experiments can be done by using only one insect. However, we have analyzed luciferin in each individual male and female fireflies. Its maximum luminescence is 575 nm (Fig. 14) with the flashing rate of 3 times/sec (Fig. 15). Mass chromatogram of sample-luciferin by LC-ESI-MS (Fig. 16, m/z 281) and its mass spectrum (ESI) (Fig. 17) were identical to the corresponding ones of the authentic luciferin.

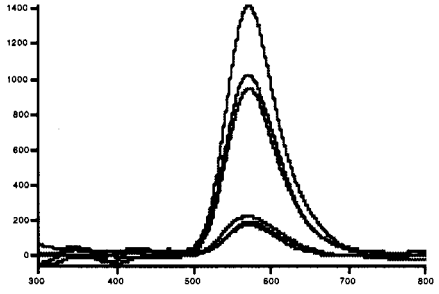


Fig. 14 Luminescence spectra of Malaysian firefly.

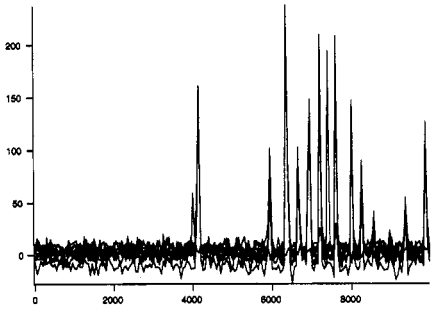


Fig. 15 Luminescence pulse

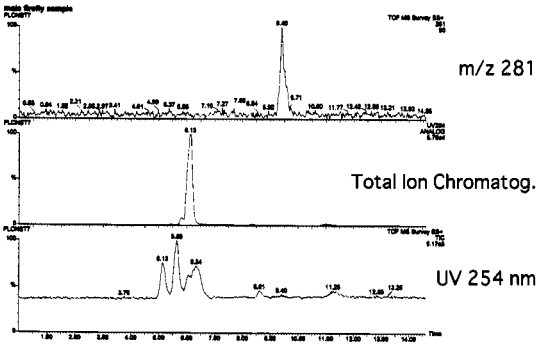


Fig. 16 LC-Mass chromatogram of firefly luciferin

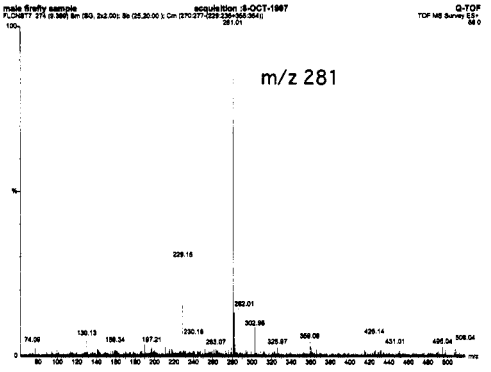


Fig. 17 Mass spectrum of firefly luciferin

Very low amount of luciferin can be determined by a single photon counter system equipped with immobilized luciferase and flow injection of a 10 µl sample. The 10^{-16} mole of luciferin/10ul is the detection limit so far. The sample extract of a firefly luminous organ for injection by LC-MS was further 1,000 times diluted in the measurement by the immobilized luciferase system (Fig.18). We have also applied this luminescence system for the assay of protein phosphatase activity. For example, (Scheme 3)

employing synthetic luciferin phosphate as substrate for protein phosphatase; thus, it cleaves off the phosphate and gives free luciferin which is detectable by our established "immobilized luciferase system". This method successfully allowed us to study the inhibition of protein phosphatase type 1 and 2A activity by either okadaic acid or tautomycin (Fig. 19).[3,7,16]

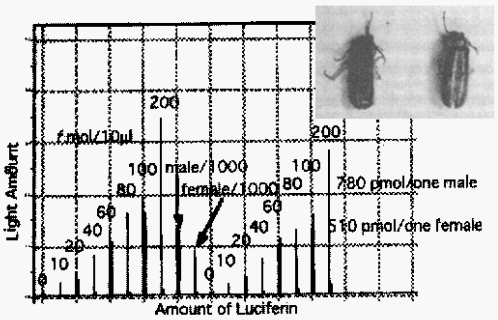


Fig. 18 Amount of luciferin detected by the immobilized luciferase.

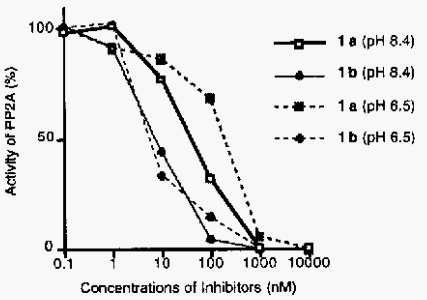
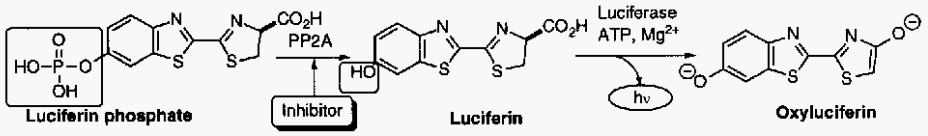


Fig. 19 An example of assay of inhibited activity of protein phosphatase type 2A (1a = tautomycin, 1b = diacid).



Scheme 3 Action of phosphatase on luciferin phosphate giving free luciferin which is detected by an immobilized luciferase system.

Application of bioluminescence for assay of protein phosphatase inhibitors

Okadaic acid (OKA) from marine sponge, *Halichondria okadai,* often contaminates in seafoods and actually it is a cause of diarrheatic poisoning. It has been identified as a protein phosphatase (type 2) inhibitor exhibiting tumor promotion.[13,14] Recently, tautomycin (TTM) has been reported to have such inhibitory action on protein phosphatase (type 1). We became particularly interested in the relationship between the molecular shape of these compounds and their inhibitory activity, for which we needed a rapid assay system and the modified bioluminescence assay could serve this purpose. We achieved the total syntheses of OKA (Fig. 20) in 1986 and TTM (Fig. 21) in 1995, and have been looking forward to doing a molecular design contributing to the field of elucidating the molecular mechanism of protein phosphatase.[15,17,18]

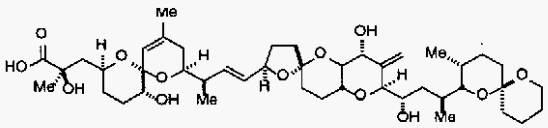


Fig. 20 Okadaic acid

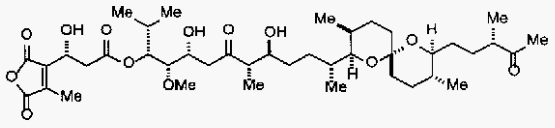


Fig. 21 Tautomycin (anhydride being non active form)

A hybrid molecule of the left-side TTM and the right-side OKA (Fig.22), OKM (so-named okada-mycin), in fact showed strong inhibitory activity by luminescence assay. Takai demonstrated with this hybrid OKM by using radioactive-labeled phosphoric protein and concluded that this inhibitor has strong inhibition to the phosphatase type 2 instead of type 1 because of the change of the right-side segment.[19]

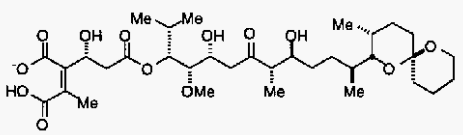


Fig. 22 Synthetic hybrid okadamycin (OKM).

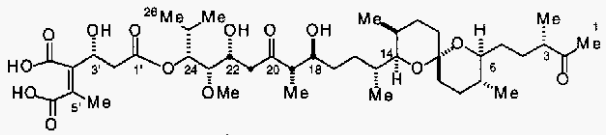


Fig. 23 100% ^{13}C enriched synthetic tautomycin at the 18, 19, 21 and 22 positions.

Information from other synthetic (ca. 10 different) analogs of TTM, we have concluded that (i) dicarboxylate is indispensable for activity, (ii) 22-OH is important, (iii) the whole molecular shape has folding at the middle of the molecule. These requirements to maintain the molecular function and shape of TTM have made its molecule be very similar to the x-ray crystal structure of OKA. We have analyzed the molecular shape of OKA in solution by NMR and molecular mechanic calculation to obtain a similar molecular shape as shown in Fig. 24 (left). The same method was employed to TTM diacid in aqueous solution to reveal that the outlook of the molecular shapes of TTM is similar to the one of OKA including the carboxylic acid anion charge, important hydroxyl group function.

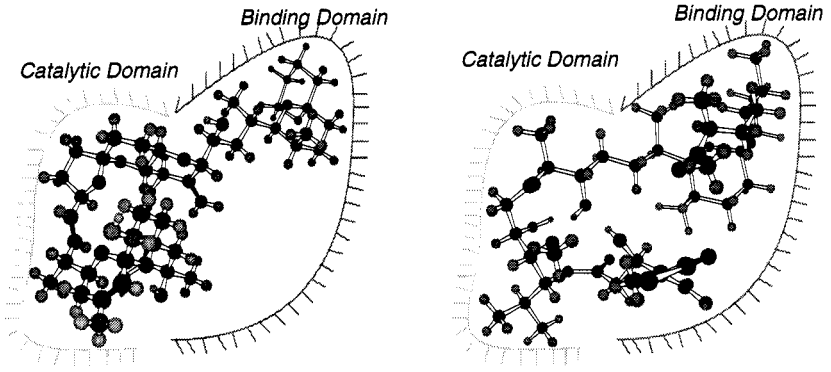


Fig. 24 3D Conformation of okadaic acid (left) and tautomycin (right) based on NMR data (*J* values and distant information) and molecular mechanic calculation.

In water solution tautomycin existed in several molecular conformations as judged from so far analysis by ^{1}HNMR.[16] We became more interested in its reactive molecular shape with protein phosphatase to see either one of those conformers will be predominant staying with the protein. An example is drawn in Fig. 25, which includes 100%-^{13}C enriched carbon atoms in tautomycin molecule at the C18, 19, 21 and 22 positions, respectively (Fig.23). This enrichment allowed us to know the *J3* coupling constants of carbons not only among the enriched atoms but also with the natural abundance, e.g. $J_{22\text{-}25}$. Assembling these data, we obtained the conformation as the following, which is still observed in protein and is now studied in progress.

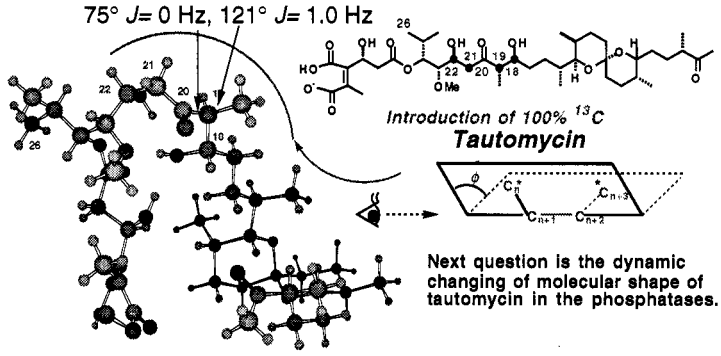


Fig. 25 Synthesis of 4-position ^{13}C enriched tautomycin and a precise conformation through ^{13}CNMR.

Conclusion

New bioluminescence system has a potential difference in molecular mechanism based on the molecular-molecular interaction between the small organic molecule and biomacromolecule. These studies aimed to get solutions in this field through organic chemistry, bioorganic and biochemical aspects. The inhibitory activity of okadaic acid/tautomycin to protein phospatases was measured by bioluminescence technology using immobilized luciferase (from cloning) and luciferin phosphate as substrate to phosphatase (both type 2A and type 1).

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