A TRIBUTE TO THE CONTRIBUTIONS OF PROFESSOR KOJI NAKANISHI IN HETEROCYCLIC CHEMISTRY

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This special issue is dedicated to Professor Koji Nakanishi for the pre-celebration of his 75th birthday. On this occasion, I would like to express my great esteem for Professor Nakanishi who, over so many years, has labored so hard in the work of this field. Although it is beyond my power, I would be most happy to contribute a summary of his distinguished achievements in the latest scientific development, i.e., structural studies of bioactive molecules and design of highly sensitive spectroscopic/chemical methods. Professor Nakanishi is an active centennial professor of Columbia University in New York and still one of the most productive chemists in the world. He has been also noted as the best magician among chemists.

For many years, Professor Nakanishi has been a leader in the isolation and structure determination of biologically important natural products. He has designed new advanced methodology for structure determination on submicroscale of diverse complex organic molecules. The essence of his work is to design combined spectroscopic/chemical derivatization methods of high sensitivity, thus making it possible to study natural products and bioactivity beyond the conventional limit imposed by the minuscule quantity of material or complexity of the problem. This trend is manifested in all his studies which has solved the most challenging problems that had eluded clarification for many years. The isolation and chemical/structural methods he developed have

provided new tools for the study of natural products and has promoted them to a higher level. He has made outstanding and pioneering contributions in a variety of spectroscopic techniques.

I. Spectroscopic methods.

His name (KN) became known through publication of his IR monograph (question/answer format) in 1962. He co-authored a book on CD (1983) summarizing the basics and theory of exciton chirality CD, a method he started in 1969 with N. Harada (his former student, Professor of Tohoku University); he also co-authored and co-edited a book covering various aspects of CD (1994) and edited a book on NMR in the lecture format (1990). The methods developed by him have greatly expanded the feasibility limit of structural and mechanistic studies of bioactive molecules.

a. Nuclear Overhauser effect (NOE) and other NMR measurements: KN performed the first pioneering applications of the NMR phenomenon widely known today as NOE in natural products structure determinations (ginkgolides, 1967). While Anet and Bourne published the first paper on NOE (1965), KN independently encountered this effect during the same time, and demonstrated its enormous potentiality for structure determination of complex organic molecules. This gave rise to new spectroscopic methods and application of general applicability, especially for targets which cannot yet be solved by X-ray crystallography.

b. Second derivative UV and FTIR difference spectroscopy (1985): The technique of second derivative UV and FTIR difference spectroscopy, exploited for the first time during structural studies of mitomycine / DNA adducts has proved to be a highly sensitive tool for microscale structural studies.

c. The exciton chirality circular dichroic method (ECCD) (1969-present): KN pioneered and developed the exciton chirality circular dichroic method,

which is based on the nonempirical coupled oscillator theory developed in the 1930s. First introduced as the dibenzoate chirality method for1,2-glycols (1969), it has continued to make progress, especially in recent years, so that it has now become applicable to almost any type of compound, cyclic, acyclic, and even biopolymeric.

The crucial finding of major theoretical (1997) and practical importance was made that entire CD curves can be represented by pair-wise addition of all interacting chromophores. Namely, the CD of a multichromophoric system, independent of whether the chromophores are identical (1981) or different (1986), is represented by summation of the CD of all pairwise interacting chromophoric systems (1983). Interaction of difference chromophores are especially important because in this case, the entire CD curve becomes characteristic for that compound (as in IR). No exception was found in 90 cases studied (1990). The bichromophoric CD approach has been extended to determine the relative and absolute configurations of flexible acyclic 1,2- and 1,3-polyols, and mixed 1,2-1,3-polyols and aminopolyols containing five contiguous chiral centers (1992-). The pairwise additivity principle enables their configurations to be determined by 1- or 2-step derivatizations. A number of new red-shifted chromophores have been devised for derivatizations of hydroxyl and primary amino groups (1992-); these chromophores can be used for natural products with pre-existing chromophores since the red shift prevents the introduced chromophores from coupling to the original chromophores; the red-shifted chromophores also enable one to use ECCD without interference from contaminants frequently present in biochemical preparations.

The first application (1985) of second derivative CD disclosed that when the immunosuppresive drug, rapamycin, binds to its binding protein (FKBP), a subtle change in drug conformation leading to increased triene planarity occurs. It is this triene moiety that is involved in the binding of the rapamycin FKBP complex

to the elicitor protein, a requirement for immunosuppression. Importantly, it was found that this change is restricted to active rapamycin derivatives. Such minuscule changes would have been difficult, if not impossible, to be detected by NMR or X-ray. Membrane bound sphingolipids are regulators of cell function and exert an extreme variety of important activities; the CD of derivatized sphingolipid establishes the relative and absolute configuration (1991).

Using porphyrins as chromophores (their intense Soret bands, ca. 420 nm, ε 350,000), his group has succeeded (1995) in securing strong coupling between chromophores 40-50 Å apart. The ECCD method outlined above leads to a vast unexplored area with exciting possibilities for bioscience, e.g., biopolymer conformation, internal interaction, molecular association. Absolute configurations of a single chiral center (1996-) can be determined by using the stacking properties of porphyrins; this will extend application of CD to unexplored areas.

MO calculations of CD and UV/VIS curves (1995-). Excellent agreement is seen between experimental and theoretical CD and UV/VIS curves of highly complex systems. This success vastly enhances the role of the exciton chirality method since it is possible to estimate the conformation of multichromophoric systems from CD curves.

Fluorscence detected exciton coupled CD (1997-). This method, a recent achievement (J.G. Dong, Ph.D. thesis, 1997) enhances the sensitivity of exciton coupled CD by 50 to 100-fold. Undoubtedly this finding will have a profound effect on future deveopment of the CD method including applications dealing with biopolymers.

d. CD-pKa measurements to determine adduct linkage points: When two chromophores are spatially close, they give rise to intensely coupled CD (ECCD). This is the case with the d-guanosine (dG)/ benzpyrene adduct (1976). Since the UV of dG changes with pH, the coupled CD spectrum also changes with pH. This change allowed measurement of the pKa of the d-G moiety, which in

turn disclosed that d-G was attached to the aromatic hydrocarbon moiety through the primary amino group. The pK could neither be measured by titration owing to the limited sample quantity, nor by UV owing to the domination of spectra by the strong non-dissociating hydrocarbon chromophore; in contrast, the intense CD required solutions more dilute than for UV measurements.

e Sequencing of sticky membrane by tandem mass spectrometry (MS):

This is a method with a 100-fold sensitivity of conventional peptide Edman degradation that allows sequencing without chromatographic separation. The transmembrane segment of many membrane proteins, exemplified by retinal proteins, are amphiphilic. Consequently, the peptidic fragments resulting from enzymatic or chemical cleavage are sticky and notoriously difficult or impossible to separate by conventional chromatographic methods. By using a tandem four-sector mass spectrometer, his group has succeeded in detecting nine out of ten peptides (the single undetected fragment is a minor tetrapeptide). This was the first case of a membrane protein being sequenced (partially) without separation of the peptides (1995).

II. Retinal proteins and other receptors. (1975-)

Starting from the early 1970s, KN has clarified numerous mechanistic and structural aspects of retinal proteins by studying the effect of incorporating over 100 synthetic retinal analogs into the apoprotein. Meny analogs have been sent to laboratories world-wide, making analog studies one of the most powerful tools in investigating the extremely complex systems involved in visual transduction: phototaxis, ion transport, etc. External point charge model for the retinal proteins has helped the understanding of the participation of electrostatic interactions within protein binding sites in regulating the absorption maxima of various pigments (1979). The "opsin shift" concept, introduced to indicate the influence of the protein binding site on the maxima of pigments, is now a general

term used without reference. The incorporation of retinal analogs into the photoreceptor of the monocelluar eukaryotic alga, *Chlamydomonas*, has shown that, contrary to general belief, isomerization of all-trans to 13-cis retinal is responsible for phototaxis. His group has also succeeded in cloning and expressing the genes of five photoreceptor rhodopsins of the goldfish (1993). Studies with pigments reconstituted from retinals in which double bond isomerizations are prevented, proved that 11-cis/all-trans and all-trans/13-cis isomerizations are essential for the activation of, respectively, rhodopsins and *Halobacterium halobium* pigments. Many other photophysical and physiological problems have been solved by using these locked analogs. Human vision sensitivity spans a range of 10^7, whereas the pupil area can only adjust the luminance 16-fold. This large difference, termed bleaching adaptation, is little understood. Incorporation of a retinal analog with a 11-cis-locked structure into the rod cells of the tiger salamander led to restoration of bleaching adaptation, and thus provided the first chemical tool for investigating the mechanism of that phenomenon (1990, 1994).

Isomerization of the rhodopsin chromophore from 11-cis to all-trans in visual transduction was established in the early 60's by G. Wald. The critical problem regarding the triggering process had, however, remained unsolved. After ten years of unpublished investigations, KN's group has succeeded in elucidating this process through enzymatic assays and spectroscopic studies of rhodopsin analogs, including one that incorporated a retinal analog with a fixed 11,12-ene through a bridging 8-membered ring. The results show that the process requires a complete isomerization, involving the full and rigid polyene system and the methyl groups of 11-cis-retinal (1994).

His group has determined critical structural aspects pertaining to the disposition of the retinal chromophores in rhodopsin and bacteriorhodopsin. In the case of rhodopsin, photoaffinity labeling has established the location of the retinal chromophore in the initial resting stage: the ionone ring is close to one of the

seven helices (helix F) and is centrally located (1994). Furthermore, the incorporation of two retinal analogs with a 11, 12-single bond into opsin and analysis of the bisignate CD curve according to exciton theory has determined the absolute sense of twist of the12,13-bond in the chromophore (1997). These two findings are critical for calcifying the chromophore/opsin tertiary structural changes accompanying the visual transduction process; in connection with these studies, his group has succeeded in preparing 3-diazo-4-oxo-11-cis-retinal, the molecule needed for liquid nitrogen photoaffinity studies, through a unique chemoenzymatic route (1997).

III. Isolation and structure determination of bioactive factors.

Althouth the main focus of KN's research efforts has shifted towards clarification of the interaction between bioactive molecules and receptor biopolymers, the isolation and structural studies of bioactive molecules have resulted in major contributions throughout his career. The majority of compounds that have been handled are noncrystalline, available only at the microgram level, and unstable. It is those characteristics that have provided the major impetus for the development of new isolation methods, and of the spectroscopic techniques which are mentioned further on. Some representative examples among the more than 180 bioactive compounds characterized by the Nakanishi group are mentioned below.

Pristimerin (1962): Triterpene, the first member of a widely used ethnobotanical antibiotic. **Taxinines** (1963-66): Structural studies led to the first derivation of the taxane skeleton which is the basis of a number of extremely important substances, such as taxol. **Chromomycins** (1963-1967): Elucidation of the structure of chromomycins and four new 2,6-deoxysugars represented one of the earliest examples of the application of exhaustive NMR decoupling. These antibiotics were an important clinical anticancer drug. **Ponasterones** (1966): The

first insect/ crustacean molting hormone to be isolated from plants; 6 kg of *Podocarpus nakaii* leaves gave 10 g of ponasterones, which were 10-fold more active than 20-hydroxyecdysone, the natural hormone. Until this finding, the amount available from insects and crustaceans was extremely limited. The large number of phytoecdysoteroids which KN and T. Takemoto et al. have isolated is now close to 100, and this has had a major impact on insect physiology. **Ginkgolides** (1967): During the course of the structural elucidation of these complex pentacyclic compounds from the fossil tree *Ginkgo biloba*, the power of the intramolecular nuclear Overhauser effect (NOE) in structural studies was first demonstrated. The ginkgolides have since been found to be potent inhibitors of platelet activating factor. The main active ingredient in the > 2 billion $ of *G. biloba* extract sold annually is ginkgolide B. **1-Methyladenine** (1969): Meiosis inducing substance (MIS) from starfish. This has played a critical role in understanding the physiology of starfish maturation. **Fluorescent "Y base" in phenylalanine RNA** (1971): Y base and congenors are the only tricyclic nucleic acid bases known. The unprecedented structure, including its absolute configuration was determined on a total of 0.3 mg, and verified by synthesis. **Antheridiogen** (1971): The first fern antheridia formation factor to be characterized. **Insect Antifeedants** (1975-1982): Starting with the characterization of warburganal from a local herb at the International Center of Insect Physiology and Ecology, Nairobi, Kenia (KN was one of the founding members of ICIPE in 1969), his group characterized many plant constituents that deter insect feeding, thus popularizing the concept of antifeedants as a means of pest control. **Benzpyrene/DNA adducts** (1976): The determination of the full structure of this first polyaromatic hydrocarbon/DNA had a major impact in clarifying the carcinogenicity of polyaromatic hydrocarbons and environmental carcinogenesis. **Q* Nucleosides** from rabbit liver tRNA (1976): Minor base in the tRNAs of animals, notably found in hepatoma cells. Studies, performed on 0.6

mg, showed that they were the first nucleosides to contain sugars, galactose and mannose, in a complex. **Mitotic hormone, trigonelline** (1978): Cell proliferation regulator, the first factor characterized that arrests the cell cycle at the G2 stage. In the early days of ^{13}C NMR, it took 9 days to measure the spectrum of this hygroscopic factor (0.25 mg) isolated from 16,000 cotyledons of garden peas. **Brevetoxin B and A** (1981-): These neurotoxins from dinoflagellates, the red-tide toxins, was the first member of molecules with a stiff ladder-like skeleton. Biosynthetic studies showed that their biosynthesis is unique. Their mode of action was regarded to be specific binding to voltage sensitive sodium channels, but his group's finding (1995) that the toxin itself efficiently transports Na^+ through membranes requires rechecking of this mechanism. **Cabenegrins** (1982): A unique antidote against the venom of a common snake in the Amazons. Injection of 1 mg/kg of cabenegrins into dogs, either 15 min before or after administration of 3 times the lethal quantity of the venom, reverses all of its effects. **New Phytoalexins** (1983-1984): Sweet potato roots treated with spore suspensions of black rot fungus, other fungi, or $HgCl_2$ produced ten new compounds related to ipomeamarone with fungicidal and antigermination activities, thus showing that inoculation or injury provides intriguing means of producing new antifungal agents. **Mitomycin C/DNA adduct** (1983-1987): Characterization of several adducts, including the long-sought cross-linked DNA adduct, clarified the mode of action of this clinically important drug. Differential second derivative UV and FTIR methods were developed during these studies. **Pavoninins, mosesins, pardaxins**, diffense secretion of certain soles (1984-1986): Pavoninins and mosesins are saponins whereas pardaxins are amphiphilic 33-peptides. All have been synthesized. The amphiphilic nature of the mixtures led to challenges in purification. **Tunichromes** (1985-): They are air- and water-sensitive pigments from tunicates, which concentrate vanadium and iron from the sea 1-10 million fold.

His group succeeded in characterization, synthesis, and clarified their interaction with vanadium. **Amphikuemin** (1986): This causes anemone fish and sea anemone symbiosis; first marine synomone to be identified. The isolation/ characterization involving tedious assay monitoring was performed 0.048 mg; synthesis accomplished. **Andrimid** (1987): Highly potent and species-specific antibiotic isolated from an intracellular symbiotic microorganism of a brown planthopper, a rice pest. This unique dipeptide has been characterized and synthesized. Its discovery suggested that symbionts may represent untapped new sources of bioactive compounds. **Crustacean ecdysone biosynthesis inhibitors** (1987-1992): The ecdysone biosynthesis inhibitor (EBI) present in crustacean eye-stalks, and elusive for many years, was isolated after a multi-year assay-monitored study. This was identified as 3-hydroxy-L-kynurenine, which is transformed in the molting gland to xanthurenic acid, the active factor; it inhibits *in vivo* hydroxylation of cholesterol to ecdysone. **Philanthotoxin** (1987-): This noncompetitive inhibitor of nicotinic acetylcholine (nACh) and glutamate receptors, responsible for muscle contraction and to memory /Huntington disease/Alzheimer diseas, respectively, was isolated from the killer wasp venom. Over 100 analogs have been synthesized. Photoaffinity labeling and structure activity relation studies have led to a tertiary structural model showing the binding of philantotoxin in the channel of the nACh receptor (1995). **Sporogenic psi factors** (1990-1992): Six endogenous psi factors (precocious sexual inducer) leading to premature sporulation were isolated from the culture medium of an ascomycetous fungus; some have been synthesized. **14-Hydroxy-4,14-retro-Retinol** (1991-): This endogenous growth factor of various mammalian cells is linked to selective gene action and differentiation and is a new secondary messenger that may have an intracellular receptor. **Ocular age A2-E** (1995-): With age, fluorescent granules accumulate in the retinal pigment epithelium. This is the leading cause of blindness in elderly people, and no

remedy exists for it. The structure of the major pigment in the granules, A2-E, isolated from the aged eyes was established by synthesis and led to revision of an earlier structure proposed by Eldred. A2-E becomes a potential target for devising cures for this disease.