CHAPTER 2: REVIEW OF LITERATURE

POLYENE MACROLIDE ANTIBIOTICS

An Overview

Polyene macrolide antibiotics are characterized by the presence of a large macrocyclic lactone ring containing a series of conjugated double bonds and one or more sugar residues. Polyene antibiotics have little or no antibacterial activity but are potent antimycotics, which are used extensively to treat systemic and non-systemic mycoses. The polyene antibiotics are produced almost exclusively by *Streptomyces* spp. Nearly one hundred polyene antibiotics have been described but some of these may not be unique or even pure as a result of the scarce evidence provided by many authors (Hammond 1977).

Most of the polyene antibiotics are among the most effective antifungal agents known, but inherent problems concerning their stability, solubility, absorption, and severe toxicity by parenteral routes have greatly limited their use in clinical practice. Since these antibiotics are far less toxic when administered by the oral or topical routes, all commercially produced polyene macrolides are therefore used primarily for treatment of topical or oral-gastrointestinal fungal infections (Schaffner 1984).

The polyene antibiotics are divided into five subgroups based on the conjugated double bonds in the polyene macrolide ring, which produces a strong chromophore with sharp UV absorption maxima. These subgroups consist of 3, 4, 5, 6, and 7 double bonds in the chromophore and are named the following: trienes, tetraenes, pentaenes, hexaenes, and heptaenes (Hammond 1977). A second way to classify polyene antibiotics is by the presence or absence of a glycosidically linked carbohydrate. This carbohydrate moiety is hexosamine mycosamine (3-amino-3,6-dideoxy-D-mannopyranose) for all cases except perimycin (Hamilton-Miller 1973).

The majority of tetraenes contain a macrolide ring of 25 to 27 carbon atoms. Natamycin and etruscomycin can be classified separately from the "classical" tetraenes due to the presence of an oxirane ring. This second ring system makes this group, the epoxide tetraenes, unique among the polyene antibiotics. Nystatin has a much larger macrolide ring of 37 carbons. Nystatin resembles the structure of the heptaenes amphotericin B and candidin and has little structural

affinity to natamycin or etruscomycin. Nystatin is classified as a tetraene due to the UV absorption of its four conjugated double bonds, but it also contains an additional two conjugated double bonds separated from the four by a methylene bridge. Nystatin consists of two components named nystatin A_1 and A_2, with nystatin A_1 having considerably more stability than A_2 (Hammond 1977).

The heptaenes can be separated into three subgroups based on the presence or absence of an aromatic side-chain. The two heptaenes of clinical importance, amphotericin B and candidin, contain no aromatic moiety (Hammond 1977). Amphotericin B remains the golden standard for the treatment of many invasive fungal infections (Viviani et al. 1998).

Thirty-seven years after the discovery of nystatin in 1950 as the first member of the polyene macrolides, the absolute stereochemistry was still only known for one member, amphotericin B. The small amount of stereochemical information about these natural products unfortunately hinders a better understanding of their structure-activity relationships. The complete stereostructure of mycotoxins A and C were elucidated in 1987 (Lancelin et al. 1988). The complete stereostructures of nystatin A_1 and natamycin were determined in 1989 and 1990, respectively. Figure 1 shows the complete stereostructures of amphotericin B (Mechlinski et al. 1970), nystatin A_1 (Lancelin and Beau 1989), and natamycin (Lancelin and Beau 1990a, 1990b).

NATAMYCIN

Discovery and Origin

Natamycin is a polyene macrolide antibiotic, which was first isolated in 1955 in the Gist-brocades research laboratories from the fermentation broth of a culture of *Streptomyces natalensis* (Stark 2000). Struyk et al. published a paper in 1957 announcing the discovery and properties of pimaricin, the original name of natamycin. The *Streptomyces* species was isolated from a soil sample obtained near the town of Pietermaritzburg in Natal, a province of South Africa (Struyk et al. 1957-1958). Therefore, the strain was named *Streptomyces natalensis* and the antifungal it produced called pimaricin (*Pi*eter*mari*tzburg). However, a World Health Organization (WHO) regulation later stated that antibiotics produced by *Streptomyces* had to carry names ending in "-mycin." The microorganism responsible and the product were to

describe their mutual connection in the names. As a result, the name "pimaricin" was not acceptable to the WHO and it had to be changed to "natamycin," which means "from *Streptomyces natalensis*" (Raab 1972).

In 1959, American investigators isolated an antibiotic from the culture medium of a *Streptomyces* strain, which originated from a soil sample collected in Chattanooga, Tennessee. Therefore, this strain was named *Streptomyces chattanoogensis* and the antibiotic it produced called tennecetin (Raab 1972). However, analytical studies and biological assays of tennecetin revealed that it was identical to the already well-known natamycin (Divekar et al. 1961). As a consequence, the name tennecetin was no longer used.

Natamycin is also identical to the antimycotic referred to in literature as antibiotic A-5283. The U.S. registered trademark of the American Cyanamid Company for natamycin is Myprozine® (Clark et al. 1964). *Streptomyces costae*, isolated from a soil sample collected in the surrounding of Madrid, Spain, has also been found to produce natamycin (Cañedo et al. 2000).

Production

Industrial production of natamycin occurs by fermentation using either *Streptomyces natalensis* or *Streptomyces gilvosporeus* (Brik 1981). The Royal Netherlands Fermentation Industries, Ltd., which later became a division of Gist-brocades, N.V., owns the original patent and the American Cyanamid Corporation owns the second patent. Both patents describe the same principle method of natamycin production. In both cases, natamycin is extracted from the culture broth by using butanol, after acidification to pH 3.0. Several purification steps including precipitation, extractions, and spray drying are then performed on the crude preparations (Raab 1972).

In the fermentation broth of *S. natalensis*, natamycin is mainly present in the crystalline form due to its low water solubility. Insoluble crystals in the form of needles or discs are formed during fermentation. Typically, particles have a diameter of 0.5 to 20 μm. A mixture of larger and smaller particles is preferred for most applications. The fermentation time, usually between 48 and 120 hours, depends on the amount of cells in the inoculum, the medium composition, and the desired yield. The fermentation is performed at temperatures between 25 and 30 °C and at pH 6 to 8 (Stark 2000).

After completion of fermentation, natamycin is recovered using extraction, filtration, and drying processes. The pH of the culture broth is adjusted first in order to solubilize the natamycin. An organic solvent, such as methanol or butanol, is then added. Filtration is used to separate the biomass and other impurities from the natamycin product. The pH is then readjusted to about neutral, which causes precipitation of natamycin crystals. After a drying step, an almost pure white powder is obtained (Stark 2000). Natamycin is now commonly produced with an activity of about 950 μg/mg (Raab 1972).

A recent patent by Gist-brocades B.V., now DSM Anti-Infectives, describes an alternative method to recover natamycin (Raghoenath and Webbers 2000). Natamycin can be separated from the fermentation broth without an organic solvent when the particle size of the biomass is reduced. The biomass of the *Streptomyces* organisms used in natamycin production consists of clusters of mycelia. The biomass is disintegrated after fermentation by homogenization or high shear mixing. Separation of natamycin from the biomass is then accomplished by using gravity gradient separation techniques. After separation, the natamycin suspension may undergo a drying process to obtain a powder (Raghoenath and Webbers 2000).

Physical and Chemical Properties

In its solid state, natamycin is present in its common crystalline form as a trihydrate. It is a creamy-white powder that is odorless and tasteless (Stark 2000). Upon heating, crystals of natamycin decompose at a temperature of approximately 200 °C. A precise melting point cannot be determined (Raab 1972). A second crystalline form has been observed as a fine birefringent powder with very small dimensions of less than 1 μm. This new modification, called the β-polymorph, is a more stable form and also has 3 moles of crystal water (Brik 1994).

Natamycin is a member of the polyene macrolide group of antifungal antibiotics. It is classified as a tetraene antibiotic since its structure contains a chromophore of four conjugated double bonds (Raab 1972). Oroshnik and Mebane (1963) inferred that the tetraene chromophore is all-*trans* on the basis of the ultraviolet and infrared spectra. The UV spectra of the tetraene chromophore displayed that the peak of the longest wavelength, which is also the narrowest, is either the strongest in the group or only slightly weaker than the second peak, indicating an all-*trans* system.

Structural studies of the polyene macrolide antibiotics by strictly chemical methods have resulted in great difficulties. Patrick et al. proposed the first structure for natamycin in 1958 with the molecular formula of $C_{34}H_{49}NO_{14}$. In 1964, after extensive reinvestigations, which included the isolation of the molecular framework as a saturated hydrocarbon, Ceder and coworkers proposed the molecular formula of $C_{33}H_{47}NO_{14}$ (Ceder et al. 1977). This structure was once again revised two years later by Golding et al. (1966) who proposed the molecular formula of $C_{33}H_{47}NO_{13}$ in which the C8 OH group is absent. Natamycin is the first polyene macrolide whose correct covalent structure, without regard to stereochemistry, was established (Golding et al. 1966). Natamycin has an empirical formula of $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.75 (Brik 1994).

Figure 1 shows the complete structural formula of natamycin. A large 25-carbon atom lactone ring, which gives natamycin its classification as a macrolide antibiotic, is connected to a mycosamine moiety by an ether linkage (Raab 1972). Ceder et al. (1977) used NMR spectroscopy to deduce that the mycosamine ring is pyranoid with a chair conformation; the hemi-ketal is 6-membered and occupies a chair conformation with the protons at C11, C12, and C13 all in axial positions; the olefinic protons H2 and H3 and the epoxy protons H4 and H5 both are *trans* to each other; and the antibiotic is diastereomerically pure. The mycosamine moiety is β-glycosidically bound to the aglycone as indicated by the anomeric coupling constant.

The structure of natamycin between C7 and C16 is identical to the structure of amphotericin B between C11 and C20 (Ceder et al. 1977). Both contain a mycosamine moiety with identical structure and stereochemistry (Brown and Sidebottom 1981). Brik (1994) describes the structural relation of natamycin to several other tetraene antibiotics. Lancelin and Beau (1990) defined the complete stereostructure of natamycin as represented in Figure 1. The configuration was established as 4R, 5R, 7S, 9R, 11S, 12R, 13S, 15R, 25R, and 1'R. The D series of the mycosamine sugar and the all-*trans* extended C16-C23 tetraene was confirmed (Lancelin and Beau 1990a, 1990b, 1995).

Many polyene antibiotics, including natamycin, are amphoteric, meaning they possess one basic group and one acidic group. These compounds will be electrophoretically neutral between pH values of approximately 5 to 9. The presence of mycosamine confers a basic charge on the molecule, with the pK of the amino group being about 8.6. The pK values for the carboxyl group of the polyene antibiotics are not well known; a value of 4 to 4.5 has been reported for

nystatin (Hamilton-Miller 1973). Natamycin possesses a carboxyl group that is present as a zwitterion (Thomas 1976). The isoelectric point of natamycin is at pH 6.5 (Raab 1972).

The end of the natamycin molecule containing both mycosamine and the carboxyl group is very polar, while the opposite end is very non-polar (Figure 1). The side of the macrolide ring that contains the tetraene chromophore is rigid and completely hydrophobic. The other side of the macrolide, which contains three hydroxyl groups is flexible and has a hydrophilic and hydrophobic face, rendering it amphipathic (Thomas 1976).

The International Union of Pure and Applied Chemistry (IUPAC) chemical name for natamycin is 22-(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.$0^{5,7}$]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid. The Chemical Abstract Service (CAS) registry number for natamycin is 7681-93-8.

Solubility

Natamycin shows good solubility in polar organic solvents and very poor solubility in water. In some cases, the presence of a small percentage of water actually increases the solubility of natamycin in organic solvents, such as n-butanol. While glacial acetic acid, methylpyrrolidone, dimethylformamide, dimethyl sulfoxide, glycerol, and propylene glycol have been listed as good solvents (Raab 1972), it is important to note the stability of natamycin in each solvent. For example, natamycin is soluble in alkaline and acidic solutions but the compound is rapidly decomposed in such solvents (Brik 1981). Clark et al. (1964) and Brik (1981) have compiled data on natamycin solubility in several important solvents. Natamycin is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol, and various oils (Struyk et al. 1957-1958).

The United States Pharmacopeia (USP) and National Formulary (NF) indicate general descriptive and solubility properties as listed in Table 1. Natamycin is listed as practically insoluble in water, slightly soluble in methanol, and soluble in glacial acetic acid and dimethylformamide (1999). The solubility data of natamycin in water show large discrepancies between different sources as listed in Table 2. These discrepancies are attributed to the increasing purity of natamycin samples, formation of solvates, and different test methods. Recent samples are less soluble in various solvents when compared to original samples, which contained a much larger percentage of impurities. In methanol, a natamycin concentration of 15

mg per mL may initially dissolve, but after the spontaneous crystallization of the solvate, only 3.3 mg per mL remains in solution (Brik 1981). In Table 2, the outlying observation of Marsh and Weiss (1967) was calculated on a weight basis from an evaporation residue. Brik (1981) used a multiple crystallized natamycin reference standard, allowed equilibration with a solvate, if present, and then analyzed the filtrate by differential spectrophotometry.

The structure of natamycin reveals important features about its solubility properties. One side of the macrolide ring has three free hydroxyl groups, while the opposite side of the ring contains the unsaturated tetraene chromophore. The hydroxyl groups and the zwitterionic carboxyl group cause the relative insolubility of natamycin in organic solvents. The presence of a six-membered ketal ring structure also contributes to the exceptional insolubility of natamycin (Thomas 1976). The tetraene system is thoroughly hydrophobic, while the remaining portion of the molecule is hydrophilic. Solubility decreases with an increase in the hydrophobic nature of the chromophore from the tetraenes to the heptaenes (Hammond 1977).

Solutions of polyene antibiotics can be carefully diluted with water, since visual precipitation does occur at concentrations of less than 50 mg/L, but they exist as micellar suspensions in aqueous media (Thomas 1976; Oostendorp 1981). The heptaene amphotericin B has been shown to disobey the Beer-Lambert law on the basis of its circular dichroism and UV absorption spectra. These properties indicate that amphotericin B exists in a relatively labile aggregated form in aqueous solution. Light-scattering measurements in aqueous solutions of amphotericin B suggest an aggregated system of about 2,000 molecules (Rinnert et al. 1977). The UV absorption spectra of amphotericin B in aqueous solution is highly degraded in contrast to the strong vibrational fine structure of amphotericin B in a dispersive medium, such as 1:1 ethanol/water mixtures (Ernst et al. 1981). Aggregation of molecules affects the free oscillation of electrons along the chain of the conjugated double bonds and results in spectral degradation, which is most severe in the fine structure (Schaffner and Mechlinski 1972).

The degree of dispersion of natamycin in water appears to be much greater than amphotericin B, approaching closer to molecular dispersion or true solution. Natamycin very precisely follows the Beer-Lambert law for its UV absorption spectra and has no apparent degradation of its strong vibrational fine structure in aqueous solution (Koontz 2001). Natamycin also has a water solubility that is more than one magnitude greater than that of amphotericin B (Schaffner and Mechlinski 1972).

An attempt to solubilize natamycin by cholate-formation, similar to the solubilization of amphotericin B by sodium deoxycholate, provided very limited success (Korteweg et al. 1961). Improvements in the aqueous solubility of natamycin can be achieved by complex formation with boric acid and by chemical modification, including the formation of alkyl esters, amides, or *N*-glycosyl derivatives (Brik 1981).

SS-natamycin is a water-soluble complex of approximately 66% natamycin and 34% of a modified polysaccharide. A clear 5% solution could be easily prepared that had a pH of approximately 8.0. SS-natamycin was only one-third as biologically active compared to natamycin. This reduced activity is not completely accounted for on a weight for weight basis, since the theoretical natamycin content is approximately 66%. The intravenous LD_{50} of SS-natamycin for rats and mice was 40 mg/kg body weight. The acute LD_{50} value of 250 mg/kg was only one-sixth the value of natamycin. The high toxicity of SS-natamycin was further confirmed in subchronic toxicity studies (Korteweg et al. 1961). The high toxicity of SS-natamycin relative to natamycin has rendered this formulation unsuitable for application in humans (Raab 1972).

An *N*-alkyl semisynthetic derivative of natamycin was synthesized which has a water solubility of greater than 30 mg/mL, representing a more than 500-fold solubility increase relative to natamycin (Suloff 1999). This natamycin derivative, *N*-(3'-*N*-dimethylaminopropylsuccimido) natamycin, was found to be less biologically active than natamycin when present in an equimolar concentration (Suloff 2002).

Stability

The stability of natamycin is affected by extreme pH values, light, heat, oxidants, chlorine, and heavy metals. The stability of natamycin is greatly dependent upon the stability of the tetraene chromophore of the molecule. Inactivation of natamycin in solution occurring by heat, light, or oxidation always begins with the cleaving of these four conjugated double bonds (Raab 1972).

In its trihydrate form, natamycin is a stable compound when it is protected from light and moisture. Storage of the dry powder at room temperature for several years only resulted in a few percent loss of activity (Brik 1981). The stability of dry natamycin is decreased when mixed in formulations with sodium chloride, citric acid, or sucrose (Raab 1972). The stability of

natamycin in aqueous suspensions is as high as in the dry powder. An aqueous suspension of 0.5% natamycin at pH 6.5 with 0.1% w/v polyoxyethylene monooleate remained stable after 24 months of storage in the dark (Clark et al. 1964). Visible or infrared irradiation resulted in nearly no inactivation of the antimycotic (Dekker and Ark 1959).

Neutral aqueous suspensions of natamycin can tolerate a temperature of 50°C for several days with only a slight decrease in activity (Stark 2000). Heating solutions and suspensions of natamycin to the boiling point did not result in any loss of activity. Only a moderate reduction in biological activity was observed after heat sterilization for 20 minutes at 110 °C (Struyk et al. 1957-1958). During a sterilization period of 30 minutes at 116 °C, a 5% aqueous suspension of natamycin shows only a slight degradation. However, after 22 hours of heating at 116 °C no antimycotic activity was detected. The preservative benzalkonium chloride did not affect natamycin stability during heat sterilization (Alsop et al. 1984).

Solutions of natamycin are quite stable at pH values between 5.0 and 9.0 when stored in the dark. Raab (1972) infers that aqueous suspensions of natamycin are more stable than aqueous solutions. Natamycin is rapidly inactivated at extreme pH ranges (Raab 1972). A 5% w/v aqueous suspension of natamycin at pH 1.5 completely lost its biological activity after storage in the dark for 2 months at room temperature or for 2 weeks at 40 °C (Brik 1976).

At low pH values, degradation of natamycin produces mycosamine and at least three additional inactive compounds. These compounds all contain intact lactone rings and have been differentiated into an amphoteric, an acidic, and a non-ionic substance. The mycosamine moiety is split off by hydrolysis of the glycosidic bond, forming an unstable aglycone that reacts with either a second molecule of aglycone or with an intact natamycin molecule. In both cases dimmers with a triene group are formed, instead of a tetraene, and the epoxy group is hydrolyzed to a diol. Decarboxylation of the aglycone is favored upon heating at low pH. (Brik 1976).

At high pH values, the lactone is saponified, forming the biologically inactive natamycoic acid. At pH 12, this saponification reaction occurs within a few hours. Additional decomposition of natamycin occurs in strongly alkaline conditions due to a series of retroaldol reactions. The long-chain heavily unsaturated aldehyde, 13-hydroxy-2,4,6,8,10-tetradecapentaene-1-al, is derived from the tetraene portion of the macrolactone ring (Brik 1976). Acetone, acetaldehyde, and ammonia were also reaction products detected (Brik 1981).

Photo-oxidation of natamycin by UV irradiation resulted in the most significant loss of biological activity. The polyene antibiotics were relatively stable to oxidation in air, but lost biological activity upon incubation with iron salts. Photolytic oxidation clearly poses a problem in the handling and storage of the polyene antibiotics (Gutteridge et al. 1983).

Oxidation of natamycin proceeds more readily at higher temperatures. Oxidative degradation of natamycin occurs more readily in organic solvents or alkaline conditions than in aqueous solutions of pH 5 to 7 (Raab 1972). An oxidative process is involved in the inactivation of natamycin when a disc saturated in a 200 ppm aqueous solution of sodium natamycin is slowly dried. Oxidation was prevented by ascorbic acid, hydroquinone, gallic acid, and a 1,000 ppm concentration of chlorophyllin (Dekker and Ark 1959). The antimycotic activity of natamycin is rapidly destroyed when exposed to chemical oxidants, such as peroxides, organic acid anhydrides, perchlorates, persulfates, permanganates, iodates, bromates, hypochlorites, and sulfites. The complete inactivation of natamycin results within two days when 0.1% sulfite is added to a saturated aqueous solution (Clark et al. 1964). Several metal ions, especially Fe(III), Ni(II), and Cr(III), promote oxidative inactivation of natamycin. The addition of complexing agents, such as EDTA or polyphosphates, can prevent this metal promoted oxidation (Brik 1981).

Thoma and Kübler (1998) have performed photostability tests on various antimycotics using a xenon lamp with a UV filter that models the spectrum of natural sunlight. The polyene macrolide antibiotics are very light sensitive antimycotic substances (Thoma and Kübler 1998). The photolysis of natamycin in methanolic solution is very rapid. After a 10 second exposure, more than 10% of the natamycin was destroyed, and after 10 minutes, the antimycotic is completely degraded. The photolytic reaction follows pseudo-first order kinetics with the acceleration constant, $k_{obs} = 0.48\ min^{-1}$. Therefore, natamycin is an antibiotic with extremely high light sensitivity. HPLC separation of natamycin after a 20 second exposure to light shows five degradation products with UV spectra of a tetraene structure, and two degradation products with typical triene UV spectra. After a short time of light exposure, the primary degradation products that are present contain the tetraene chromophore. With prolonged light exposure, photoproducts with UV spectra of trienes are formed (Thoma and Kübler 1997). An irradiated solution of natamycin did not show the presence of free mycosamine (Narasimhachari et al. 1967).

Photodegradation of drugs is greatly influenced by the wavelength of absorbed light. Thoma and Kübler (1996) have shown that the photodegradation of natamycin in methanolic solution is dependent on the wavelengths of UV irradiation exposure. Dekker and Ark (1959) described the rapid inactivation of natamycin caused by UV irradiation primarily with wavelengths between 300 to 350 nm. The addition of sodium potassium chlorophyllin prevented inactivation of natamycin by UV irradiation. Chlorophyllin has considerable absorption in the UV wavelengths between 300 and 400 nm, which covers the region where natamycin is most sensitive to destruction (Dekker and Ark 1959). The photostabilization principle of spectral overlay is often applied successfully. Substances that absorb light in a similar wavelength range as the photolabile compound exhibit good spectral overlay. For example, the drug, terconazole, is protected from light by excipients showing good spectral overlay of the absorption spectrum of the drug (Thoma and Kübler 1998). Propylgallate also protected natamycin against damage caused by UV irradiation (Gutteridge et al. 1983).

Dekker and Ark (1959) report that the inactivation of sodium natamycin by UV irradiation is different from the inactivation by oxidation. The extinction peaks of the UV absorption spectrum of natamycin disappeared after loss of antimycotic activity due to oxidation, but were only slightly reduced in intensity after inactivation by UV irradiation. A conjugated polyene antibiotic that is closely related to natamycin, named rimocidin, has been used to propose a mechanism for degradation upon exposure to UV irradiation. The UV absorption spectrum of rimocidin indicates that upon exposure to UV irradiation a transition from a biologically active *trans*-configuration to an inactive or less active *cis*-configuration occurs. Dekker and Ark (1959) indicate that this *cis-trans* isomerization may also apply to natamycin based on its UV absorption spectra.

Photosensitization is the process of light energy being absorbed by a chromophore and this energy is then passed on to other molecules, which lack the ability to absorb at the excitation wavelength (Taylor and Radda 1971). Sensitizers such as methylene blue, eosine, *p*-aminoacetophenone, and acetophenone accelerate the photolytic degradation of natamycin (Narasimhachari et al. 1967). Flavins, such as riboflavin, can also act as photosensitizers (Taylor and Radda 1971). In darkness, riboflavin has no effect on the absorption spectrum of natamycin (Hendriks and Berends 1958). Irradiating an aqueous solution of natamycin with visible light of a wavelength of 443 nm did not result in any degradation. In the presence of riboflavin or

lumichrome, visible light was shown to cause destruction of natamycin in aqueous solutions as evidenced by a rapid decrease in the absorbances of the natamycin maxima at 304 and 319 nm. This effect is surprising, since natamycin does not have any absorption in the visible region; it appears that riboflavin or lumichrome absorbs the light and causes the destruction of pimaricin in some manner. Oxygen showed a strong inhibiting effect on the photodegradation of the riboflavin-natamycin and lumichrome-natamycin systems, which suggests a direct transfer of the excitation energy from riboflavin or lumichrome to natamycin without free radicals being involved (Zondag et al. 1960).

Posthuma and Berends (1960) proposed a triplet-triplet transfer as the mechanism of the photodynamic destruction of natamycin in the presence of riboflavin. The following reaction sequence has been proposed:

$$\text{Riboflavin} + h\nu \longrightarrow \text{Riboflavin}^* \text{ (singlet)} \longrightarrow \text{Riboflavin}^* \text{ (triplet)} \qquad \text{(A)}$$

$$\text{Riboflavin}^* \text{ (triplet)} + \text{Natamycin} \longrightarrow \text{Riboflavin} + \text{Natamycin}^* \text{ (triplet)} \qquad \text{(B)}$$

In reaction A, riboflavin is first excited by a 445 nm light quantum to its singlet state and then a singlet-triplet transition occurs by conversion of the spin moment. From its triplet state, an energy transfer to a natamycin molecule is possible if the triplet energy of natamycin is lower than that of riboflavin. Reaction B is allowed by spin-conservation rules and the overall result of this triplet-triplet transfer is an excited natamycin molecule. A very sensitive compound like natamycin with its reactive tetraene structure is likely decomposed if it is excited to its triplet state (Posthuma and Berends 1960, 1966). Triplet quenchers, such as ascorbic acid and hydroquinone, inhibited the riboflavin-sensitized photochemical destruction of natamycin (Hendriks and Berends 1958; Posthuma and Berends 1966). Paramagnetic ions also inhibited the transfer of the excitation energy between riboflavin and natamycin (Posthuma and Berends 1961).

Mode of Antimicrobial Action

In 1958, two observations were made which proved important to the understanding of the mechanism of action of polyene antibiotics. Nystatin was found to damage the membranes of yeast in some manner that allowed low molecular weight material to leak from the cell but larger molecules to be retained. The addition of sterols to the growth medium was found to allow protection of fungi against polyene antibiotics (Hammond 1977). The polyene antibiotics were

believed to function by the same basic mechanism of action as indicated by the available evidence (Kinsky 1967). The antifungal activity of polyene antibiotics is primarily based on their ability to change the permeability of the cell membrane. Two consecutive stages are involved in the polyene mode of action: a) binding of the antibiotic on the cell membrane, and b) change in the permeability of the membrane (Raab 1972).

The polyene antibiotics have been shown to only bind to those membranes, which contain sterols. Sterols are necessary for the structure and function of membranes of the eukaryotes, including fungi, green plants, and animals. Sterols are absent from the membranes of the prokaryotes, including bacteria and blue-green algae. It is proposed that when the polyene antibiotic reaches the eukaryotic cell membrane, the strong affinity of the hydrophobic chain of the polyene for the membrane sterol draws the polyene molecule into the membrane. This allows the hydrophobic region of the polyene to lie parallel to the sterol ring. The breaking of phospholipid-sterol interactions creates instability in the membrane and introduces the hydrophilic polyol surface of the polyene into the interior of the membrane. The presence of a hydrophilic chain into the membrane may account for the polyene-induced permeability changes observed. Several sterol-polyene complexes are believed to approach each other to form aggregates with their hydrophilic regions arranged to form a central hydrophilic pore in the membrane (Hammond and Lambert 1978).

The binding site of the polyene antibiotic on the cell membrane is a sterol, most likely ergosterol. However, among the different polyenes, the importance of these binding sites varies. The polyene antibiotics were classified into two groups based on *in vitro* experiments, which examined the destruction of artificial membranes composed of phospholipids and sterols in different ratios. The first group includes polyenes, which urgently need the sterols for binding on the cell membrane, such as amphotericin B and nystatin. The second group contains those, which are far more independent of sterol compounds, such as natamycin and etruscomycin. Natamycin is most closely related chemically to etruscomycin. On its C25 atom, etruscomycin carries a more hydrophobic C_4H_9 group while natamycin has a CH_3 group (Raab 1972).

Etruscomycin was shown to be more effective than natamycin in growth tests. Early studies suggested that natamycin did not have any effect in altering the permeability of *Acholeplasma laidlawii* or liposomes. The action on liposomes was found to be a function of the nature and concentration of the sterol. Natamycin at 30-40 μg/mL caused the release of K^+ from

Saccharomyces cerevisiae. Natamycin induced the release of K^+ when the cholesterol and ergosterol content was increased to equal that found in the *S. cerevisiae* membrane. The length of the hydrophobic chain has been suggested as important in determining sterol preference (Gale 1984).

The early work suggested that the toxicity of polyene antibiotics was related to molecular size, but more recent studies have shown that selectivity lies in the more subtle differences in amphiphilicity, charge, and side-chain structure (Gale 1984). Novák and Novák (1980) stated that the effect of natamycin on inhibiting growth may not be explained unambiguously by fungicide activity, which emphasizes the differences in the mechanism of action of polyenes. In a comparative study with other polyenes, natamycin differed in its effects on cell size and shape, overall ion leakage, and potassium and sodium movements of nystatin sensitive and resistant mutants of *Candida albicans* (Novák et al. 1983). Novák et al. (1983) concluded that although natamycin has strong activity against *C. albicans*, it cannot induce ion movements even at concentrations 10 times greater than its minimum inhibitory concentration (MIC) value. In contrast, these ion movements occur in the presence of nystatin and to a lesser extent with amphotericin B. In general, the polyenes do not share a common mechanism of action. With polyenes that are able to induce ion leakage, the mechanism cannot be simply the formation of physical pores, which leads to passive solute leakage (Novák et al. 1983).

Spectrum of Activity

Natamycin possesses strong antifungal properties, yet it is not active against bacteria. Struyk et al. (1957) reported that the majority of yeast and fungi are inhibited by natamycin concentrations of between 1 and 10 μg/mL. Natamycin is highly active against young, dividing fungal cells but it does not kill spores (Oostendorp 1981). Raab (1972) has compiled an overview of the activity of natamycin against dermatophytes, other fungi, yeasts, and yeast-like organisms, with an emphasis on those organisms important in human medicine.

Natamycin acts as a unique food preservative since it is an effective inhibitor of all target organisms without causing negative effects on product quality. It has a much broader spectrum of activity against yeasts and molds than any other fungicide allowed for use in the food industry. Natamycin is especially useful for products that require natural bacterial ripening processes since it does not inhibit bacteria (Stark 2000). Bacteria are not susceptible to

natamycin since their membranes lack sterols, and therefore, reported MICs of natamycin against bacteria are high (>10,000 μg/mL) (Pedersen 1992). Klis et al. (1959) showed that a natamycin concentration of 10 ppm completely inhibited 16 common food spoilage yeasts and molds by an agar plate method. The polyenes, amphotericin B and nystatin, required concentrations of 100 ppm or greater to completely inhibit all of these spoilage fungi (Klis et al. 1959). Bullerman (1977) demonstrated that 5 ppm of natamycin prevented growth of all the mold isolates from natural cheese, the majority of which were *Penicillium* spp., at 12 and 5 °C for at least 7 days. At 25 °C, which is the optimum growth temperature for most molds, the growth of 96% of the mold isolates was prevented for at least 7 days (Bullerman 1977).

The sensitivity of most mold strains, including many *Aspergillus* and *Penicillium* spp., is at concentrations of less than 10 ppm. Yeasts possess an even greater sensitivity to natamycin than molds. It is important that natamycin is active at such low concentrations, since it is practically insoluble in aqueous solution. Only natamycin dissolved in solution has antimycotic activity, therefore, its low solubility may be a factor limiting this activity (Stark 2000). Natamycin appeared to be more effective at inhibiting growth of *Aspergillus ochraceus* in liquid media than in olive paste (Gourama and Bullerman 1988).

Natamycin has been observed to have an inhibitory effect that is greater on toxin production than on growth for all of the toxigenic molds. In studies using laboratory media, a 1 ppm concentration of natamycin had little inhibitory effect on growth of *Aspergillus flavus* but inhibited toxin production of aflatoxin B_1 by 25%. Natamycin inhibited mycelial growth of *A. ochraceus* by 16 to 52% at concentrations of 1 to 50 ppm, but at 10 ppm ochratoxin production was completely inhibited. Penicillic acid and patulin production was strongly inhibited at all test concentrations (Ray and Bullerman 1982). At a level of 20 ppm natamycin, mycelial growth of *A. ochraceus* was inhibited 80 to 100% and production of penicillic acid was completely inhibited. There was no stimulation of growth or penicillic acid production by subinhibitory concentrations of natamycin. Natamycin was observed to inhibit penicillic acid production more effectively than mycelial growth (Gourama and Bullerman 1988). Natamycin at concentrations between 5 and 20 μg/mL sometimes inhibited *Apergillus parasiticus*, but did not prevent growth and production of aflatoxins. In the presence of subinhibitory concentrations of natamycin, *A. parasiticus* is able to overcome the initial inhibition and produce large amounts of aflatoxins B_1 and G_1 (Rusul and Marth 1988).

Resistance and Tolerance

The problem of polyene resistance is nonexistent from a clinical viewpoint. A similar statement cannot be made about any other antibiotics in common therapeutic use (Hamilton-Miller 1974). The Joint FAO/WHO Expert Committee on Food Additives (1976) expressed general concern about the use of therapeutic agents in food, but it agreed that the data on natamycin demonstrated that problems were unlikely to arise from microbial resistance.

Natamycin has been used in the food industry for decades but resistance has never been observed (Stark 2000). After several years of continuous use of natamycin in cheese warehouses, no molds or yeast were found to be insensitive to natamycin. This may be partly attributed to the strong fungicidal activity of natamycin and its instability, which inhibits accumulation of intact natamycin. None of the 26 mold strains isolated from cheese warehouses became noticeably less sensitive to natamycin after 25-30 transfers to media with increasing natamycin concentrations (De Boer and Stolk-Horsthuis 1977).

Tolerance to natamycin developed gradually when attempting to induce *Candida albicans* resistance by serial passages on Sabouraud maltose agar. After 25 serial passages, the MIC of *C. albicans* increased from 2.5-12.5 μg/mL to 12.5-50.0 μg/mL (Výmola and Hejzlar 1970). Resistance to natamycin may be induced with difficulty under laboratory conditions by mutagenesis in fungal cells either containing decreased amounts of ergosterol or lacking ergosterol. Mutant strains that replace ergosterol with another sterol are not able to survive in nature (Stark 2000).

Analysis

UV Spectrophotometry

UV spectrophotometry, using methanol with 0.1% acetic acid as the solvent, is a useful assay method for routine control. The acetic acid acts as a "wavelength stabilizer" since small amounts of alkali introduce a small red shift of up to 2 nm. The UV spectrum of natamycin exhibits sharp maxima at 290, 303, and 318 nm with a shoulder at 280 nm and a broad maximum at 220 nm. The absorption spectrum between 280 and 320 nm is characteristic of the all-*trans* tetraene segment; the maximum at 220 nm is recognized as the en-one chromophore (Brik 1981).

The highest degree of correlation between biological activity and tetraene content is obtained with differential spectrophotometry. This method is based on the measurement of absorbance at

the main maximum at 303 nm and at the minima at 295 and 311 nm. This baseline method for the determination of natamycin in pharmaceuticals is calculated in equation 1 (Brik 1981):

$$E = E_{303} - \frac{1}{2}(E_{295} + E_{311}) \quad (1)$$

In cheese extracts, the matrix interferes at the wavelengths necessary for analysis, making the pharmaceutical baseline method unsuitable. However, differential spectrophotometry can be effectively employed on the portion of the spectrum with higher wavelengths that is not obstructed. This substitute method uses the straight line between the absorbance at 311 and 329 nm as the baseline. The difference between the absorbance at the maximum at 317 nm and this baseline is used to calculate the net absorbance in equation 2 (De Ruig et al. 1987):

$$E = E_{317} - \frac{2}{3}(E_{322}) - \frac{1}{3}(E_{329}) \quad (2)$$

This spectrophotometric method is used as the international standard for determining the natamycin content of cheese rind and of the cheese adjacent to the rind (ISO 1991; IDF 1992).

It is of great importance to emphasize that this method is not suitable for stability studies due to the lack of correlation between biological activity and tetraene content upon degradation. Oxidative inactivation of natamycin results in the disappearance of the UV absorption spectra, but inactivation by UV irradiation causes only a slight reduction in the intensity of the absorption spectrum (Dekker and Ark 1959). A 5% natamycin suspension that is completely degraded at pH 1.5 results in a spectrophotometrically calculated concentration that is too high due to a small amount of inactive tetraene (aglycone). Complete inactivation of a 5% natamycin suspension occurs within a few hours at pH 12, but spectrophotometrically there is no apparent decrease in the tetraene content due to the formation of the biologically inactive tetraene natamycoic acid (Brik 1981).

HPLC

The greater selectivity of HPLC makes it a useful method for the stability testing of natamycin (Brik 1981). Some polyene antibiotics, such as nystatin, are not defined single substances but a mixture of various substances (Thoma and Kübler 1998). Intact natamycin appears as a single peak on an HPLC chromatogram. Therefore, relative to other polyenes, natamycin is considered a purer compound (Thoma and Kübler 1997). Brik (1994) has

summarized several HPLC methods, which have been used for the determination of trace amounts of natamycin in cheese. Natamycin is typically analyzed with a C8 or C18 reverse phase column and detected by UV spectrophotometry at 303 nm or photodiode array.

HPLC allows the separation of degradation products from intact natamycin. These degradation products have a shorter retention time (De Ruig 1987). Thoma and Kübler (1997) reported the separation of seven photodegradation products of natamycin by HPLC. The HPLC method used as the international standard for determining the natamycin content of cheese uses a mobile phase of methanol-water-acetic acid, 60:40:5, v/v/v. Methanol is also the solvent used to extract natamycin from cheese and cheese rind. However, this standard mentions that natamycin is unstable in aqueous methanol (De Ruig et al. 1987; ISO 1991; IDF 1992). More recent reports have stated that solutions of natamycin in aqueous or pure methanol are stable (Fletouris et al. 1995; Capitán-Vallvey et al. 2000).

In Vitro Susceptibility Testing (MIC)

Antifungal susceptibility testing *in vitro* is commonly characterized by a poor standardization and reproducibility of most of the methods employed. These difficulties can be explained by many variables, including inoculum size, temperature, incubation duration, and medium composition, which affect the outcome of *in vitro* testing (Kobayashi and Medoff 1977; Buchta and Otcenásek 1996). A great disparity in reported MICs can also occur when using the same fungi, the same antimycotics and test concentrations, identical inocula, incubation temperatures and duration, and media, but varying the method of testing (Rinaldi 1993). Therefore, if susceptibility data from different laboratories is to be compared, strict standardization conditions must be followed (Kobayashi and Medoff 1977).

The standard fungal strain for the biological assay of natamycin is *Saccharomyces cerevisiae* ATCC 9763. Agar dilution series are recommended to determine the sensitivity of yeasts and molds to natamycin. A stock solution of natamycin is prepared by dissolving natamycin in 100% methanol; working standards are prepared by dilution of this stock with sterile distilled water. The influence of the solvent methanol is removed by preparing media without natamycin but with the appropriate concentrations of methanol. Methanol concentrations lower than 2.5% are not required to be examined (Raab 1972). Several concentration levels of the natamycin are incorporated in agar medium and fungi are inoculated onto the agar surface. After incubation,

the presence or absence of fungal growth is compared to an inoculated, natamycin-free control and a MIC can be determined (Fromtling 1987).

The advantages of the agar dilution assay are that many fungal strains and test compounds can be tested at the same time and heterogeneous populations of test organisms can be tested and identified. Important disadvantages include poor solubility and distribution of the antibiotic in the agar medium, inability to obtain minimum fungicidal concentration values, and poor correlation with broth dilution data with some groups of compounds (Fromtling 1987).

Proton NMR

De Bruyn et al. (1978) obtained a 300 MHz ^{1}H NMR spectrum of natamycin in alkaline trifluoroethanol-d_3. Due to extensive overlap and insufficient resolution, the chemical shifts of the olefinic protons H17 to H22 could not be discriminated (De Bruyn et al. 1978). Ceder et al. (1977) described the 270 MHz ^{1}H NMR spectrum of *N*-acetyl natamycin in d_5-pyridine. The tetraene protons and methylene protons at C6, C8, C14, and C24 are not completely separated (Ceder et al. 1977). The spectra of natamycin in trifluoroethanol-d_3 and *N*-acetyl natamycin in pyridine-d_5 both exhibit chemical shifts that are widely divergent from those observed for natamycin in dimethyl sulfoxide-d_6 (DMSO-d_6). A complete set of double resonance experiments was conducted to obtain assignment of protons for natamycin in DMSO-d_6 (Brown and Sidebottom 1981). Lancelin and Beau (1995) measured the *J* coupling constants for all protons of natamycin in methanol-d_4 with the exception of the C18 to C21 segment because of a chemical shift degeneration of the proton resonances. These measurements were performed at 300 MHz using either the double quantum filtered COSY spectra or resolution-enhanced 1D spectra. The best resolution for the olefinic protons of the C16 to C23 tetraene portion was shown by the double quantum filtered COSY spectrum of *N*-acetyl natamycin in DMSO-d_6. The coupling constants $J_{20,21}$ were the only that remained undetermined (Lancelin and Beau 1995).

Toxicology

Struyk et al. (1957) presented the first acute toxicity data on natamycin for rats, mice, and guinea pigs. The acute intraperitoneal toxicity of natamycin was found to be only one-tenth of that of nystatin based on a data comparison with literature. Although, the oral toxicity of natamycin is two to three times greater than that of nystatin, which may be a result of better

resorption of natamycin in the intestinal tract (Struyk et al. 1957-1958). Non-absorption of natamycin was shown in 11 tests that involved the oral ingestion of 125-500 mg of natamycin and assay of the serum from eight patients, which had no natamycin activity (Clark et al. 1964).

Natamycin has a very low oral toxicity. It has a single dose LD_{50} of 2.73 g/kg for the male rat and 1.42 g/kg for the male rabbit. Feeding of diets containing 500 ppm of natamycin was well tolerated by rats over a period of 2 years. Dietary levels of 125 ppm or 250 ppm of natamycin fed to dogs for 2 years did not have any harmful effects (Levinskas et al. 1966). Natamycin has been administered orally to ten adult humans with various systemic fungal infections. Its use was limited to dosage levels of 600 to 1,000 mg per day due to nausea, vomiting, and diarrhea (Newcomer et al. 1960). No single incidence of allergy to natamycin has ever been detected in humans in either clinical applications or industrial production (Pasyk et al. 1976). The hemolytic activity of natamycin is lower than that of nystatin or amphotericin B. Degradation products from acidic conditions, alkaline conditions, or UV exposure have been shown to be less toxic than the parent compound (Noordervliet 1978; Brik 1981).

Clark et al. (1964) concluded that natamycin concentrations as high as 100 ppm are safe in food. The WHO and FAO established the acceptable daily intake (ADI) at 0.30 mg/kg (FAO and WHO 1976). The average food consumer only ingests about 0.002 mg/kg of natamycin each day from cheese and sausage products (Oostendorp 1981).

Food Applications

Natamycin has been proposed as a preservative for many food products including cheeses, sausage, and various fruits. The availability of natamycin may be a limiting factor when it is applied on solid food products. The active dissolved natamycin cannot reach the entire surface of some products, which may result in mold growth on unprotected areas (Stark 2000).

Block cheeses are typically treated with natamycin by a plastic coating that covers the entire cheese surface. This coating distributes the natamycin homogeneously over the cheese surface. On most cheeses, a coating containing 100-750 ppm of natamycin is applied three to five times (Stark 2000). Natamycin was detected in the cheese rind (about 1 mm thick) but none was detected below the rind after 12 weeks of storage. Since diffusion of natamycin is extremely small, a low concentration remains active on the cheese surface for a long duration (de Ruig and

van den Berg 1985). In rindless French cheeses, natamycin penetrates about 2-4 mm into the product (Oostendorp 1981).

The casings of dry sausages can be soaked for two hours in a 1,000 ppm suspension of natamycin to achieve a homogeneous distribution over the sausage surface. Strawberries, raspberries, and cranberries, which are sprayed with a 50 ppm solution of natamycin shortly before harvesting exhibit less spoilage. A more effective method is to dip the berries in a 10-100 ppm solution of natamycin after harvesting, which prolongs the shelf life by several days (Stark 2000).

Low concentrations of natamycin (1-5 ppm) can eliminate all yeasts and molds present in beverages and prevent spoilage during the storage period. After production, the packaging of these products is well sealed and, therefore, contamination is unlikely before the consumer opens the package. These products include juices, lemonades, soft drinks, beer, wine, and fruit yogurt (Noordervliet 1978; Stark 2000). Fresh orange juice inoculated with natural contaminants spoiled after 1 week of storage, but in the presence of 1.25, 2.5, 5, 10, or 20 ppm of natamycin, there was no spoilage during 8 weeks of storage (Shirk and Clark 1963). Natamycin was shown to be stable in orange juice when stored in the dark under refrigeration (Shirk et al. 1962).

Shredded Cheese Preservation

The very low aqueous solubility of natamycin requires its application as an aqueous suspension to the shredded cheese surface, which results in clogging of spray nozzles and a heterogeneous distribution to the cheese surface. Solubility may be a limiting factor in the bioavailability of active natamycin, since the dissolved fraction must diffuse to the site of action and bind to the target organism (Stark 2000). The application of natamycin as a dry powder mixed with an anti-caking agent, such as cellulose, likely limits the availability of natamycin to elicit an antifungal response.

During the ripening and storage of cheese products, the natamycin is decomposed (Lück and Jager 1997). Cheese products are exposed to high-intensity fluorescent lighting in the retail dairy case, until consumer purchase and storage. The UV light emitted from the fluorescent lamps impacts the cheese product through the sections of the polymer packaging that are translucent. Critical amounts of natamycin treated onto these cheese products are likely degraded by the time of purchase by the consumer.

The effect of visible light should not be neglected due to riboflavin photosensitization. In the presence of riboflavin, visible light was shown to cause destruction of natamycin in aqueous solutions (Zondag et al. 1960). Dairy products contain a high concentration of riboflavin (Borle et al. 2001). Cheddar cheese contains 3.75 mg of riboflavin per kg and low-moisture, part-skim mozzarella cheese contains 3.43 mg of riboflavin per kg (USDA 2002). Fluorescent light exposure causes degradation of riboflavin in cheese, which occurs primarily at the surface and is dependent on oxygen (Marsh et al. 1994).

Fluorescent Lighting

Supermarkets and their dairy cases are illuminated by fluorescent lamps. Fluorescent lamps consist of a filament and/or cathode, anode, conducting gas, nonconducting gas, phosphor(s), and glass bulb. The conducting gas is mercury vapor provided by addition of a drop of mercury into each lamp. Fluorescent lamps have spectral power distributions, which contain the mercury emission lines at approximately, 254, 313, 365, 405, 436, 546, and 587 nm. The glass used to manufacture the lamp affects the lower UV cut-off wavelength. The intensity of the emission lines is dependent on the amount of mercury inserted and the thickness of the phosphor layer. There is generally a large variance in the intensity of fluorescent lamps due to the difficulty in controlling the amount of mercury added during production. The intensity and spectral distribution can also vary throughout the life of the lamp (Piechocki 1998). A May 1999 report by the National Electrical Manufacturers Association states that the "estimated indoor UV exposure during one eight hour workday is equivalent to just over a minute of midday solar exposure on a clear July day in Washington, D.C."

Commercial Products for Food Preservation

Pure natamycin powder is the active ingredient in several commercial product formulations. Delvocid® Instant is a powder containing 50% natamycin and 50% lactose, which is used in many food applications. Delvocid® Dip and Premi® Nat are special powder formulations that contain a thickening agent and are designed to be applied by dipping or spraying the surface of cheese or dry sausages. Delvocid® Sol is a special formulation for large-scale use in poly(vinyl acetate) (PVA) coatings. Delvocell® combines the anticaking properties of powdered cellulose

and the antifungal activity of natamycin for primary use on shredded cheese (Stark 2000). Natamax™ is a powder containing 50% natamycin and 50% sodium chloride.

Regulatory Status

On June 22, 1982, the FDA first permitted use of natamycin on consumer cheese slices and cuts in the United States. Application was allowed by dip, spray, or other methods such as incorporation into edible or plastic cheese coatings. The recommended concentration levels ranged from 300 to 2000 ppm, depending on the type of cheese (Andres 1982). On December 1, 1998, the FDA amended the food additive regulations to provide for the safe use of a dry form of natamycin as an antimycotic in cheeses. The dry mix contains natamycin with a safe and suitable anticaking agent, which resulted in no greater than 20 ppm of natamycin in the finished product. Application of natamycin by dipping or spraying, using an aqueous suspension of 200 to 300 ppm, remained an approved method of delivery (CFR 1998). On March 8, 2001, the FDA revoked the limitations on the application of natamycin to the surface of cuts and slices of cheese and set forth that natamycin may be applied on cheese in amounts not to exceed 20 ppm in the finished product as determined by International Dairy Federation (IDF) Standard 140A:1992 (IDF 1992). Natamycin is no longer restricted in its method of application, which may include dipping, spraying, or as a dry mixture with safe and suitable anticaking agents (CFR 2001).

In most countries, natamycin is only permitted for use in the surface treatment of cheese and dry sausages. In the European Union, the maximum level of natamycin on the surface of hard, semihard, and semisoft cheese and dry cured sausages is 1 mg per dm^2 surface. Natamycin also may not be present at a depth greater than 5 mm (Stark 2000).

Medical Applications

Since natamycin is not absorbed from the intestinal tract it is primarily used for the topical treatment of fungal infections of the skin and mucous membranes. Mixed infections caused by dermatophytes and yeast-like fungi are very suitably treated with natamycin. Natamycin has established itself as one of the most effective antifungal antibiotics in topical therapy and is used in dermatology, gynecology, ophthalmology, otorhinolaryngology, pediatrics, and internal medicine (Pasyk et al. 1976). Parenteral treatment is not acceptable due to toxicity and may be associated with a large number and variety of adverse effects (Stock 1981).

Pasyk et al. (1976) describes six different pharmaceutical formulations of natamycin: Pimafucin® vaginal tablets containing 25 mg of natamycin, 1% Pimafucin® suspension, 2.5% Pimafucin® oral suspension, 1.5% Pimafucin® powder, 2% Pimafucin® cream, and Pimafucort® ointment (10 mg of natamycin, 3.5 mg of neomycin, and 10 mg of hydrocortisone per gram). A bioadhesive bilayered natamycin buccal tablet was developed, which prolonged the local release of natamycin in the oral cavity while maintaining the antimycotic concentration greater than the MIC value for *Candida albicans* (Uzunoglu et al. 2000).

Natamycin has been effective topically in the treatment of fungal infections of the eye due to its superior intraocular penetration (Schaffner 1984). In the U.S., the only approved medical application of natamycin is for treatment of fungal infections of the eye. Alcon Laboratories, Inc. (Fort Worth, TX) manufactures eyedrops under the trade name Natacyn®. Natacyn® is a sterile 5% natamycin ophthalmic suspension used to treat blepharitis, conjunctivitis, or keratitis due to susceptible fungi, including *Fusarium solani* (Parfitt 1999).

CYCLODEXTRINS AND THEIR MOLECULAR INCLUSION COMPLEXES

Introduction

Supramolecular chemistry involves all intermolecular interactions where covalent bonds are not formed between the interacting species. The host-guest interaction is the most frequent type, with an important host being the cyclodextrins. Szejtli (1998) states that the first reference of cyclodextrins was published by Villiers in 1891. Villiers called the crystalline material "cellulosine" due to its similarity to cellulose in resisting acid hydrolysis and because it did not show reducing properties. In the early 1900s, Schardinger succeeded in isolating *Bacillus macerans*, which is now the most frequently used source of the enzyme cyclodextrin transglycosylase, by which cyclodextrin is produced. In the 1930s, Freudenberg and co-workers determined the structures of the Schardinger cyclodextrins (α and β). The discovery and structural determination of γ-cyclodextrin occurred between 1948 and 1950. In the 1950s, Cramer studied the ability of cyclodextrins to form inclusion complexes with a wide range of guest molecules. After adequate toxicological studies proved that any toxicity attributed to cyclodextrins had its source from complexed impurities, an inadequate form of administration, or

extreme dosing, research in this field has exhibited a logarithmic increase (Szejtli 1998). Today, cyclodextrins are used in food, cosmetic, and pharmaceutical products, photochemical and chemical industries, biotechnology, pesticides, and for treatment of wastewater (Gous and Krige 1992; Hedges 1998).

Physical and Chemical Properties

Cyclodextrins (CDs) are cyclic oligosaccharides of α-D-glucopyranose formed by the action of certain enzymes on starch. The most common CDs are α-CD, β-CD, and γ-CD, which contain six, seven, and eight glucose units, respectively. α-CD, β-CD, and γ-CD are commonly referred to as the native or natural CDs. The chemical structures of the native CDs are shown in Figure 2. CDs containing less than six glucose units are too strained to exist and those with greater than eight units are very soluble, hard to isolate, and infrequently studied. Many important characteristics of the native CDs are summarized by Connors (1997) and Szejtli (1998).

The native CDs are crystalline, homogeneous, and nonhygroscopic compounds. The CD molecule is described as a conical cylinder or shallow truncated cone. The glucose units are linked to each other by glycosidic α-1,4 bonds. The CD can form intramolecular hydrogen bonds between its secondary hydroxyl groups. A complete secondary belt is formed by these hydrogen bonds making β-CD a rather rigid structure. This intramolecular hydrogen bond formation is the most likely explanation for the low water solubility of β-CD relative to the other native CDs. In the α-CD molecule, the hydrogen bond belt is incomplete since one glucose unit is in a distorted position. The γ-CD molecule has a noncoplanar, more flexible structure, which makes it the most soluble of the native CDs (Szejtli 1998).

The diameter of the CD cavity is larger on the side containing the secondary hydroxyl groups. The side containing the primary hydroxyls has a reduced cavity diameter due to the free rotation of these primary hydroxyls. The molecular dimensions of the native CD cavities are depicted in Figure 3. The nonbonding electron pairs of the glycosidic oxygen bridges are oriented toward the inside of the cavity, which produces a high electron density inside and imparts some Lewis base characteristics (Szejtli 1998).

The environment within the CD cavity has been inferred by some calculational studies. Lichtenthaler and Immel (1996) use solid-state complex structures to develop “lipophilicity

patterns." It was concluded that the three native CDs are very similar with the wider secondary hydroxyl end of the cavity being hydrophilic and the narrower primary hydroxyl end being hydrophobic.

The unique structural properties of the CD cavity explain some of the atypical characteristics of these molecules. The exterior of the CD molecule has numerous hydroxyl groups and, therefore, is fairly polar. The interior of the cavity is nonpolar relative to the exterior and relative to the common external environments, specifically water (Connors 1997). CDs act as host molecules to form inclusion complexes fairly nonspecifically with a wide variety of guest molecules. The only apparent requirement is that the guest molecule must fit into the cavity, even if only partially (Saenger et al. 1998).

Complex formation may be proven in solution, but the isolated crystalline dry powder is often only a very fine dispersion of the CD and the guest. However, the dissolution and reformation of the complex is an immediate process upon addition of water to this dispersion. This type of guest behavior is not a difficulty when the goal of complexation is to enhance solubility and improve bioavailability since all biological systems contain water (Szejtli 1996). CDs are not suitable mediums for microorganisms, especially yeast and mold. This is an important detail for the use of CD complexes in dusting powder preparations (Szejtli 1988). The efficiency of complexation may sometimes be rather low and, therefore, relatively large amounts of CDs must be used to complex small amounts of drug. Typically, solid drug-CD complexes contain less than 5 to 10% of the drug (Loftsson and Brewster 1996; Loftsson et al. 1999).

The number of possible CD derivatives is unlimited since the CDs contain 18 (α-CD), 21 (β-CD), or 24 (γ-CD) substitutable hydroxyl groups. The syntheses of greater than 1,500 derivatives had been published by 1997. The following CDs are produced industrially: methylated CDs, hydroxyalkylated CDs, acetylated CDs, reactive CDs, and branched CDs (Szejtli 1998). One such hydroxyalkylated CD is hydroxypropyl β-CD (HP β-CD), which is produced by the condensation reaction of β-CD with propylene oxide. HP β-CD is an intrinsically amorphous mixture of many chemical components, all of which exhibit good water solubility and good complexation ability. HP β-CD did not have any detectable oral toxicity even upon chronic administration of large doses to mice (Pitha et al. 1986). HP β-CD (2.7-5.1 DS) was shown to be harmless at concentrations up to 1 g in intravenous human administration

(Szejtli 1988). In 1997, only HP β-CD, sulfobutyl β-CD, and γ-CD were supported by satisfactory toxicological documentation as parenteral drug carriers (Szejtli 1998).

Benefits and Applications

Complexation of guest compounds with CDs provides certain benefits. Some of these benefits include: alteration of guest solubility, stabilization against the effects of light, heat, and oxidation, masking of unwanted physiological effects, and reduction of volatility (Hedges 1998).

CDs can stabilize, have no effect on reactivity, or accelerate drug degradation. The CD molecule can at least partially shield the drug molecule from attack by various reactive molecules. Although CD complexation typically increases the stability of the drug, there are examples of accelerated degradation such as the β-lactam antibiotics. The most common application of CDs in the pharmaceutical industry is to enhance drug solubility in aqueous solutions. In general, the lower the aqueous solubility of the pure drug, the greater the relative solubility enhancement gained by CD complexation. CDs prove to be useful tools to obtain aqueous drug solutions without the use of organic cosolvents, surfactants, or lipids, and to increase dissolution rates and oral bioavailability of solid drug complexes (Loftsson and Brewster 1996).

CD can interact with a product metabolite synthesized by cells. Such interaction has been found to stimulate the formation of products such as antibiotics in a small number of fermentations performed in CD-containing media. β-CD greatly stimulated the production of lankacidin C, γ-CD had an intermediate effect, and α-CD showed the least effect. In the presence of 1.5% β-CD, production of lankacidin increased from 0.22 mg/mL to 3.00 mg/mL. The lankacidins produced existed as inclusion complexes in the culture filtrate. The stimulatory effect of β-CD was observed for all *Streptomyces* species known to produce lankacidins (Sawada et al. 1987).

Preparation of Cyclodextrin Inclusion Complexes

The preparation of CD inclusion complexes is a relatively simple procedure, however, in most cases the reaction conditions must be conformed to the specific guest compound. The majority of the complex formation processes occur in aqueous solutions or at least in the presence of water. The presence of at least a minimum amount of water is necessary for inclusion processes.

Cosolvents, such as ethanol, are not required in most cases but are applied in the preparation of CD complexes with drugs and flavors (Szente 1996b).

In aqueous solutions of CDs or CD derivatives at ambient temperature, the guest is stirred or vigorously agitated until the maximum solubility is attained. In certain cases, additional agitation besides normal stirring, especially ultrasonication, has been reported to increase complexation efficiency. The remaining undissolved guest is removed by filtration or centrifugation, and the clear aqueous solution is evaporated to dryness by vacuum evaporation, spray-drying, or freeze-drying depending on the nature of the guest compound. Complexation in solution is a well-suited method for the highly water-soluble CD derivatives, such as HP β-CD. Several other techniques are used to form CD complexes including: complexation in suspension, kneading, cogrinding or mechanochemical activation, or even melting together the potential guest with CD (Szente 1996b).

Weak Interactions Involved in Complexation

The most probable mode of binding, derived from both thermodynamic and NMR studies, involves the insertion of the less polar part of the guest molecule into the cavity. The hydrophilic groups, such as hydroxyl, amino, and carboxyl, or the charged groups, such as ammonium and carboxylate, remain exposed to the bulk solvent even after inclusion of the hydrophobic segment. The simple "hydrophilic outside-lipophilic inside model" cannot always be applied, as is the case with the following guest molecules: 4-hydroxyphenethylamine, short alkanediols, and ephedrines (Rekharsky and Inoue 1998).

The formation of inclusion complexes of guest molecules with CDs in aqueous solution results in a considerable rearrangement and removal of the water molecules originally solvated to both the CD and the guest. This process also initiates the release of water molecules from the CD cavity into the bulk water. The main forces involved in binding are believed to be primarily van der Waals and hydrophobic interactions, but hydrogen bonding and steric effects also can be a factor. In general, the thermodynamic quantities for the 1:1 complexation reactions of natural and modified CDs are consistent with the hydrophobic nature of the host-guest interactions. Complex stabilities are greater for neutral compounds compared to those of the corresponding charged species derived from the same original guest molecules. Weak forces that function

locally in CD inclusion complexation are not always cooperative in nature and may act independently or even counteract one another in some cases (Rekharsky and Inoue 1998).

The flexibility of the guest molecule has an important role in the stability of CD complexes. Increasing flexibility in a guest molecule leads to a more favorable complexation entropy because there are more possible "conformers" that can fit properly into the cavity (Rekharsky and Inoue 1998).

Inclusion Complexes of Polyene Macrolides

Amphotericin B was found to form an inclusion complex with γ-CD both in aqueous solution and in the solid state. The solubility of amphotericin B can be enhanced up to 200-fold by complexation with γ-CD. The antifungal activity of the complex was found to be identical to that of the parent compound. Complexes of 1:1 and 1:2 stoichiometry were identified with stability constants of 462 and 42 M^{-1}, respectively. The solid complex showed improved dissolution and enhanced stability in solution relative to free amphotericin B (Vikmon et al. 1985; Rajagopalan et al. 1986). Rajagopalan et al. (1986) proposes a mechanism for the amphotericin B:γ-CD 1:1 inclusion complex based on the most probable site in the amphotericin B molecule susceptible to attack by hydroxyl ions at pH 12. The solubility of amphotericin B was also increased 8-fold in the presence of a 40% w/v aqueous HP β-CD solution (Loftsson et al. 1991).

Nystatin and flavofungin are able to form inclusion complexes with γ-CD, which allowed 45- and 80-fold increases in aqueous solubility, respectively. The very heat-sensitive flavofungin was protected from thermal degradation in its complexed form (Vikmon et al. 1988). The stability of nystatin in aqueous solution was improved by γ-CD complexation. Nystatin solutions at room temperature did not degrade more than 10% within 1 week of storage in the dark (van Doorne and Bosch 1991).

Erythrocytes have proved to be useful and important *in vitro* models for polyene macrolide toxicity studies. Erythrocytes possess cholesterol-rich cellular membranes and, therefore, are similar to other sensitive sterol-containing eukaryotic cells (Schaffner 1984). The hemolysis of human erythrocytes by nystatin is substantially reduced in the presence of γ-CD (Szejtli 1988).

Analytical Methods of Cyclodextrin Inclusion Complexes

Binding Constants: Definitions and Principles

A *molecular complex* is defined by Conners (1987) as a noncovalently bound species of definite substrate-to-ligand stoichiometry that is formed in a facile equilibrium process in solution. The terms *complex formation*, *complexation*, *molecular association*, and *binding* are one and the same. The *substrate*, S, is the guest whose physical or chemical properties are experimentally observed. The *ligand*, L, is the host (CD) whose concentration is the independent variable. Stoichiometric ratios are always given in the order S:L, so that a 1:2 ratio implies the complex SL_2.

The most commonly assigned stoichiometric ratio for CD complexes is 1:1, however, other ratios are known including 1:2, 2:1, and 2:2. SL, SL_2, and S_2L are the three simplest complex stoichiometries and are formed according to the following equilibria:

$$S + L \rightleftharpoons SL$$

$$SL + L \rightleftharpoons SL_2$$

$$S + SL \rightleftharpoons S_2L$$

The stepwise binding constants for these equilibria, denoted K_{11}, K_{12}, and K_{21}, are defined by equations 3-5.

$$K_{11} = \frac{[SL]}{[S][L]} \tag{3}$$

$$K_{12} = \frac{[SL_2]}{[SL][L]} \tag{4}$$

$$K_{21} = \frac{[S_2L]}{[S][SL]} \tag{5}$$

In these equations, brackets signify molar concentration so that each constant has the unit M^{-1}. The formation of higher complexes directly from the substrate and ligand can be written as

$$mS + nL \rightleftharpoons S_mL_n$$

with the *overall binding constant* β_{mn}:

$$\beta_{mn} = \frac{[S_mL_n]}{[S]^m[L]^n} \tag{6}$$

The overall binding constant can be represented as a product of the stepwise constants. In CD studies, complex stabilities are typically expressed in terms of the stepwise binding constants (Connors 1987).

Phase Solubility Diagrams (Solubility Isotherms)

Higuchi and Conners (1965) applied the solubility method to study the interaction of a slightly soluble substrate, S, and a soluble ligand, L. The experimental procedure determines the total molar solubility of substrate, S_t, as a function of total molar concentration of ligand, L_t, at constant temperature and ionic strength. Several vials are prepared, which contain equal amounts of S in great excess of its normal solubility and a solvent of constant volume containing increasing concentrations of L. The systems are brought to solubility equilibrium by shaking or tumbling at constant temperature. Equilibration time commonly takes 1 to 2 days, but longer durations of 1 to 2 weeks have been reported. The solution phase is then analyzed for S_t, most commonly by absorption spectroscopy. There should be no spectral shift in the diluted analytical solutions caused by the complex formation if absorption spectroscopy is to be used. By plotting S_t against L_t, the phase solubility diagram can be constructed (Higuchi and Connors 1965; Connors 1987).

Phase solubility diagrams can be separated into two main classes, Type A and Type B. Type A systems indicate the formation of soluble complexes between S and L. S_0 is the equilibrium solubility of S in the absence of L. This solubility of S is apparently increased with increasing concentration of L. Type A_L diagrams demonstrate a linear increase in the solubility of S with unchanged stoichiometry. Type A_P diagrams exhibit a positive deviation from linearity, which indicates a continuous increase in the stoichiometry of the complex. Type A_N diagrams show a negative deviation from linearity and indicate systems of even greater complexity. This response infers either an increase of the host ratio within the complex (1:1 to 2:1) or a change in the solute-solvent interaction (hydration, ionization of the guest) or a combination of both. Type B systems are observed when complexes of limited or decreased solubility are formed and, therefore, will not be discussed further. In many cases, the stoichiometry of the complex formed is dependent on the concentration ratios. Initially, only a 1:1 complex is formed, but at higher CD concentration the stoichiometry is more complicated (Szejtli 1996). These diagrams make it possible to learn about the complex stoichiometry and to estimate binding constants.

Determination of Stoichiometry

In order to evaluate the binding constant of a complex, the stoichiometric coefficients *m* and *n* in S_mL_n must be known. The method of continuous variation, commonly called Job's method (Job 1928), is one of several methods that are able to yield the ratio *n*/*m*. Job's method was originally developed for metal-ligand complexes, but this method has been adapted to the measurement of organic complexes, such as those formed by CDs. Ingham (1975) summarizes the important assumptions of the method of continuous variation as: (a) neither S nor L self-associate, (b) the law of mass action is obeyed, and (c) only one complex is formed.

The method of continuous variation involves preparing a series of solutions of S and L in which the sum of the total substrate concentration, S_t, and the total ligand concentration, L_t, is held constant (Connors 1987). This method has been applied using absorption spectroscopy as the analytical tool. A wavelength is chosen at which a large absorbance change is observed upon complexation. The absorbance that is observed in the absence of complexation is subtracted the absorbance of each solution containing S and L. This difference in absorbance is then plotted against x ($0 \leq x \leq 1$) to find x_{max}. The dimensionless quantity x is defined as

$$x = \frac{L_t}{S_t + L_t} \tag{7}$$

More recently, the method of continuous variation has been applied to NMR spectroscopy. Two situations may occur in NMR experiments with CD inclusion complexes: (a) the slow exchange condition and (b) the fast exchange condition. In slow exchange, the free and complexed forms of one component produce separated signals, and the ratio of the free and complexed forms can be measured directly by digital integration of the relevant signals on the NMR spectrum. In fast exchange, only shifts of the spectral lines are observed due to fast averaging by the exchange between free and bound states. With a signal belonging to S, for example, the quantity $\Delta\delta_{obs} \times S_t$ will be proportional to SL and should be plotted against x to find x_{max}. The quantity $\Delta\delta_{obs}$ denotes the chemical shift difference between free S and the observed value for a given ratio of x (Djedaïni et al. 1990).

The sharpness of the maximum of the continuous variation curve at $x = 0.5$ is dependent on the magnitude of the overall binding constant β_{11}. There is some difficulty in determining the correct values for *n* and *m* for very weak complexes since they are dependent upon an accurate value of x_{max} (Likussar and Boltz 1971). Any experimentally significant deviation of x_{max} from

0.5 may be evidence for a second complex, with the likely additional species being either the 2:1 or 1:2 complexes (Connors 1987).

Calculation of the 1:1 Binding Constant

If a single 1:1 complex is present, the mass balance expressions for S_t and L_t in the solubility experiment are

$$\begin{aligned} S_t &= [S] + [SL] \\ L_t &= [L] + [SL] \end{aligned} \tag{8}$$

[S] is constant since pure solid substrate is present in the system, so this quantity is called s_0 and the material balance on substrate is represented as

$$S_t = s_0 + [SL] \tag{9}$$

The solubility isotherm is given by combining equations 8 and 9 with the definition of the stability constant K_{11}.

$$S_t = s_0 + \frac{K_{11} s_0 L_t}{1 + K_{11} s_o} \tag{10}$$

Therefore, a plot of S_t against L_t is linear with intercept s_0 (Type A_L diagram) and slope $K_{11}s_0/(1 - K_{11}s_0)$. The stability constant can be calculated from equation 11 (Connors 1987).

$$K_{11} = \frac{\text{slope}}{s_0(1 - \text{slope})} \tag{11}$$

A slope of less than one with a Type A_L diagram does not necessarily indicate that only a 1:1 complex is formed, but this assumption is typically made if no additional information is available (Higuchi and Connors 1965). The K_{11} can be calculated from the Type A_P diagram by iteration. The value of K_{11} cannot be calculated from the Type A_N diagram (Szejtli 1996).

Strength of Cyclodextrin Complexes

Connors (1995) compiled the binding constants (K_{11}) of 1:1 complexes of α-CD, β-CD, and γ-CD with many substrates from numerous publications and performed statistical analysis. These complex stabilities appear to be reasonably described as normally distributed in log K_{11} when treated as statistical populations. The mean values of K_{11} for α-CD, β-CD, and γ-CD are 129 M^{-1}, 490 M^{-1}, and 355 M^{-1}, respectively (Connors 1995).

Proton NMR

NMR spectroscopy is the most important method applicable in solution since the driving force for CD inclusion frequently is of solvophobic nature and most CD applications involve action in a liquid matrix. The recent advances of NMR techniques has allowed a much more detailed structural elucidation of CDs and their complexes. Pharmaceutical uses of CDs for the protection or targeting of drugs currently require the structural characterization of administered compounds by law (Schneider et al. 1998).

The formation of CD inclusion complexes with guest molecules can be proven by ^{1}H NMR spectroscopy (Norwig et al. 1988). Demarco and Thakkar (1970) have demonstrated that ^{1}H NMR can provide evidence for the inclusion of aromatic substances into CDs. In general, if a guest molecule is located within the CD cavity, then the hydrogen atoms located on the inner surface of the cavity (H3 and H5) will be considerably shielded by the guest, while the hydrogen atoms on the outer surface (H1, H2, H4) will be unaffected by the inclusion complex formation (Demarco and Thakkar 1970; Wood et al. 1977). The spatial arrangement of the protons on the inside and outside of the CD cavity is displayed in Figure 4.

Many inclusion complex geometries have been proposed, which show the guest molecule penetrating into the CD cavity from the wider, secondary hydroxyl side (Schneider et al. 1998). Several barbiturates are assumed to enter the CD cavity from the narrower, primary hydroxyl side based on the relative magnitudes of the chemical shifts for H5 and H3 (Thakkar and Demarco 1971). A common structural inference is that if only H3 undergoes a shift in the presence of a guest then the cavity penetration is shallow, but if H5 also shifts the penetration is deep (Connors 1997).

Since no covalent bonds are formed or broken during the guest-CD complexation, the complexes are in dynamic equilibrium with free guest and CD molecules in the solution. The association-dissociation process between the guest and CD may be reversible and very rapid in the time period of microseconds to milliseconds. On a time-averaged basis, corresponding hydrogens on each of the glucose units will receive the same shielding effect from the included guest (Wood et al. 1977).

Most of the protons in a conventional one-dimensional spectrum can be identified with high field instruments due to the high symmetry of the CD molecule. Proton shielding differences

among α-, β-, and γ-CD are values of only 0.1 ppm at the anomeric H1 and are even less at the other positions.

Solvents have a profound influence on both the CD complexation free energies and the CD ^{1}H NMR spectra. In protic solvents such as water, intermolecular exchange between solute and solvent is too fast on the NMR time scale to observe separate OH signals. In DMSO, separate signals for the OH groups and their couplings to the vicinal C-H protons can be analyzed (Schneider et al. 1998).

NMR experiments involving the complexes of unsubstituted CDs are technically difficult because of the low solubility of the complexes in D_2O. No sufficiently high concentration can be achieved for obtaining an adequate spectrum with many complexes (Szejtli 1982). Evidence suggests that the nature of the solvent can influence or control the structure of the complex. The common solvent effect consists of a decrease in the stability of the complex relative to water (Connors 1997). The complex stability in D_2O is slightly greater than that in H_2O. The properties of liquid H_2O and D_2O are very similar to each other, but D_2O forms stronger hydrogen bonds than H_2O and there is more structural order in liquid D_2O than in liquid H_2O at equal temperature (Wang and Matsui 1994).

NMR titrations measure chemical shift changes as a function of concentration. The observed shift changes offer insight into the conformation of the formed supramolecular complexes, which is difficult to extract from other analysis such as UV-visible titrations. This method offers the advantage of providing several independent signals for the evaluation of complex stability constants. The magnitude of the upfield shifts of the CD's H3 and H5 protons has been suggested to be used as a measure of the complex stability and the depth of inclusion. Proton shifts induced by the CD on the guest are usually small due to the weak shielding tensors in the alicyclic CD structure. Such shifts are proposed to be predominantly due to changes of the microenvironment. However, these shielding effects can be of sufficient magnitude to be used for NMR titrations. The shielding effect of the CD can be used where the guest has weak shielding tensors. CD shielding can also be used to identify those segments of the guest, which reside inside the CD cavity (Schneider et al. 1998).

During NMR titration experiments with CDs, internal reference compounds such as TMS were reported to experience significant downfield shifts, which were believed to be the result of inclusion complex formation with the reference. External references were therefore

recommended to obtain more reliable data (Schneider et al. 1998). Wood et al. (1977) used a hydrogen (H1) on the outer surface of α-CD as the chemical shift reference. This H1 was chosen since complexation with *p*-iodoaniline was expected to occur by inclusion of the aromatic and would have little influence on the shielding of outer-surface hydrogens. The chemical shift of H1 was virtually unaffected by inclusion of *p*-iodoaniline when measured relative to external TSP (Wood et al. 1977). However, recent studies show that shift changes of internal references in the presence of CDs are not the result of inclusion in the CD cavity. These shift changes were attributed to changes in the magnetic susceptibilities of water due to hydrogen bonding with the glucose units of the CDs (Schneider et al. 1998).

Modification of Absorption Spectra of Complexed Guests

The shift of the UV-visible absorption maxima of a guest molecule under the influence of complexation often results in small changes. This effect may be explained by the high electron density existing inside the CD cavity, which partially shields the excitable electrons and chromophores of guests that are located within. Complexation of a guest may often result in bathochromic shifts (red shifts) and band broadening of the UV absorption and also small shifts in the molar absorption coefficient. The observed shift of the absorption maximum by several nanometers, or the decrease of the UV absorption by up to 20%, upon addition of CD to the solution of the guest, is sometimes large enough to calculate complex stability (Szejtli 1996; Szente 1996a).

The UV spectra of guests at adequately high CD concentrations are typically similar to those observed in ethanolic solutions. At low CD concentrations, the spectra appear intermediate between those in ethanolic and pure aqueous solutions. In the absence of γ-CD, amphotericin B forms colloid-like multimolecular aggregates in aqueous solutions. When γ-CD is present, true solutions can be prepared which show similar spectral properties as amphotericin B dissolved in organic solvents. The UV spectrum of a concentrated aqueous solution of the amphotericin B:γ-CD complex was shown to be nearly identical to those observed in ethanolic solution. The presence of γ-CD strongly shifts the equilibrium toward the disaggregated form of amphotericin B (Kajtár et al. 1989).

HPLC

HPLC is the most acceptable and reliable technique to determine the actual guest content of CD complexes. This method requires the complete dissolution and dissociation of the complex in a suitable solvent, most preferably in the mobile phase (Szente 1996a).

In the presence of organic solvents, the CD solubility generally decreases due to complex formation with the solvent molecules. The solubility of β-CD decreases with increasing concentrations of methanol to effectively zero in 100% methanol. However, there is a maximum on the concentration-solubility curve with ethanol, 2-propanol, acetonitrile, and tetrahydrofuran (Taghvaei and Stewart 1991; Chatjigakis et al. 1992).

Thermoanalytical Characterization

The thermal analysis of CD complexes has been used to differentiate between inclusion complexes and adsorbates, and to characterize the unique thermal effects due to molecular entrapment during a distinct, standard heating process. Most of the water molecules within the CD cavity are released at < 100 °C in the native CDs. Complexes must have a guest with a melting or boiling point below the thermal degradation range of the CD or must be volatile in the temperature range 60-250 °C to be studied by these methods (Szente 1996a). Thermal analysis has mainly been applied to demonstrate the different behavior of an inclusion compound relative to its physical mixture of component compounds (von Plessing Rossel et al. 2000).

Particle Size Distribution

The particle size of solid CD complexes can affect the dissolution rate and, therefore, the bioavailability of the product. The particle size distribution and crystalline properties of the complex are dependent on the method of complex preparation. In general, β-CD complexes of a given guest have the smallest particle sizes when they are prepared by kneading or by coprecipitation and washing with solvents to remove the adsorbed part of the guest.

Dry mode determination is generally not suitable for many active pharmaceuticals since they are too small and prone to agglomeration. A fluid dispersion is then required and a liquid medium in which the particles will not dissolve must be determined. Most active pharmaceutical ingredients have some degree of water solubility. An inert nonpolar solvent, such as mineral oil or dimethylpolysiloxane (DMPS), is commonly used in fraction cell mode. DMPS is not as

nonpolar as mineral oil, but it is available in various viscosities and is less prone to retaining bubbles. Saturated solutions are typically only used as the dispersion medium as a last resort. Laser diffraction particle size distribution analysis is a commonly used method in the pharmaceutical industry to physically characterize excipients (Barber et al. 1998).

Wettability

Wettability tests can quickly differentiate between true inclusion complexes and physical mixtures of the CD and guest. Wettability studies have indicated that formation of CD inclusion complexes with lipophilic drugs, pesticides, and flavor compounds substantially improves their wettability in water (Szente 1996a).

The wettability of solid CD complexes is commonly characterized by the following methods: observation of the upward migration of water in sample-filled tubes (Szente and Szejtli 1987), measurement of the contact angle (Bajor et al. 1988), and observation of sedimentation rates of sample powders in water. The physical mixture and inclusion complex powders must be prepared in an identical manner before testing. Samples should be equilibrated with the environmental humidity and screened through a sieve to obtain a definite range of particle size. The wetting process of the solid powders is dependent on the particle size of the samples. This particle size determines the capillary distribution of the powder in the method using the upward migration of water in sample-filled columns (Szente and Szejtli 1987).

Diffusion

The diffusion of guest molecules is reduced upon their complexation with a CD molecule, since this represents a 3 to 25-fold increase in the molecular weight (Szejtli 1988). The effect of CDs on diffusion through a semipermeable membrane is very important since absorption of biologically active molecules always occurs through such a membrane. In homogeneous solution, the diffusion rate of complexes is always lower than that of the free guest. However, in the case of guest compounds with poor solubility, the enhancement in solubility overcompensates the decrease of the diffusion rate constant. As a result, the observed diffusion rate in most cases exhibits a considerable increase (Szejtli 1996).

Food Applications

The main use of CDs in the food and cosmetic industries is for the molecular encapsulation of flavors and fragrances (Szejtli 1988). Hashimoto (1996) summarizes the major advantages of using CD complexes in food, cosmetics, and toiletries as:

"(i) Protection of active ingredients against:

(a) oxidation,

(b) light-induced reactions,

(c) heat-promoted decomposition and self-decomposition, and

(d) loss by volatility, sublimation.

(ii) Elimination (or reduction) of:

(a) undesired tastes and odors,

(b) microbiological contamination,

(c) other undesired components, and

(d) hygroscopicity.

(iii) Increasing solubilities of poorly soluble or unsoluble components.

(iv) Technological advantages:

(a) stable, standardizable compositions,

(b) simple dosing and handling of dry powders,

(c) reduced packaging and storage costs, and

(d) more economical technological processes and labor saving."

A few food antimicrobials have been studied for their ability to form CD inclusion complexes. Alkyl parabens are able to form inclusion complexes with α- and β-CD, resulting in an increased dissolution rate. Alkyl paraben molecules appear inactive as antimicrobial agents when included within the CD cavity, since they are not able to directly contact the cell membranes. The antimicrobial activity is proportional to the amount of the free alkyl paraben in aqueous solution. The MIC value of alkyl paraben increases 2- to 10-fold upon addition of CD to solution (Szejtli 1988). CD inclusion complexes of benzoic acid and dehydroacetic acid have been prepared (Hashimoto 1996).

The treatment of β-CD on separated cream proved to be an effective process for removing cholesterol from cheddar cheese and accelerating ripening (Kwak et al. 2002). It is obvious that the possible applications of CD in the food industry are quite numerous and many have been

reviewed in detail by several authors (Szejtli 1988; Hedges et al. 1995; Hashimoto 1996). Hedges (1998) has compiled a list of selected products containing CDs in the food, pharmaceutical, and cosmetic industries.

In 1998, the price of β-CD reached an acceptable level, even for the most raw material sensitive industries. The price of α- and γ-CD will always remain higher than β-CD because of both their lower yield and their lower volume of production (Szejtli 1998).

Regulatory Status

In 1997, a self-affirmed GRAS petition allowed β-CD to be used as a flavor carrier and protectant at a level of 2% in various food products in the U.S. (O'Donnell 2001). In May 2000, Wacker Biochem Corporation (Wacker Biochem) submitted to the FDA an independent GRAS determination for γ-CD as a stabilizer, emulsifier, carrier, and formulation aid. The FDA did not question the self-affirmed GRAS status and assigned it the GRAS Notice No. GRN 000046 in September 2000 (FDA 2000). In March 2001, Wacker Biochem also submitted to the FDA an independent GRAS determination for β-CD as a flavor carrier or protectant. The FDA did not question the self-affirmed GRAS status and assigned it the GRAS Notice No. GRN 000074 in October 2001 (FDA 2001).

TABLES AND FIGURES

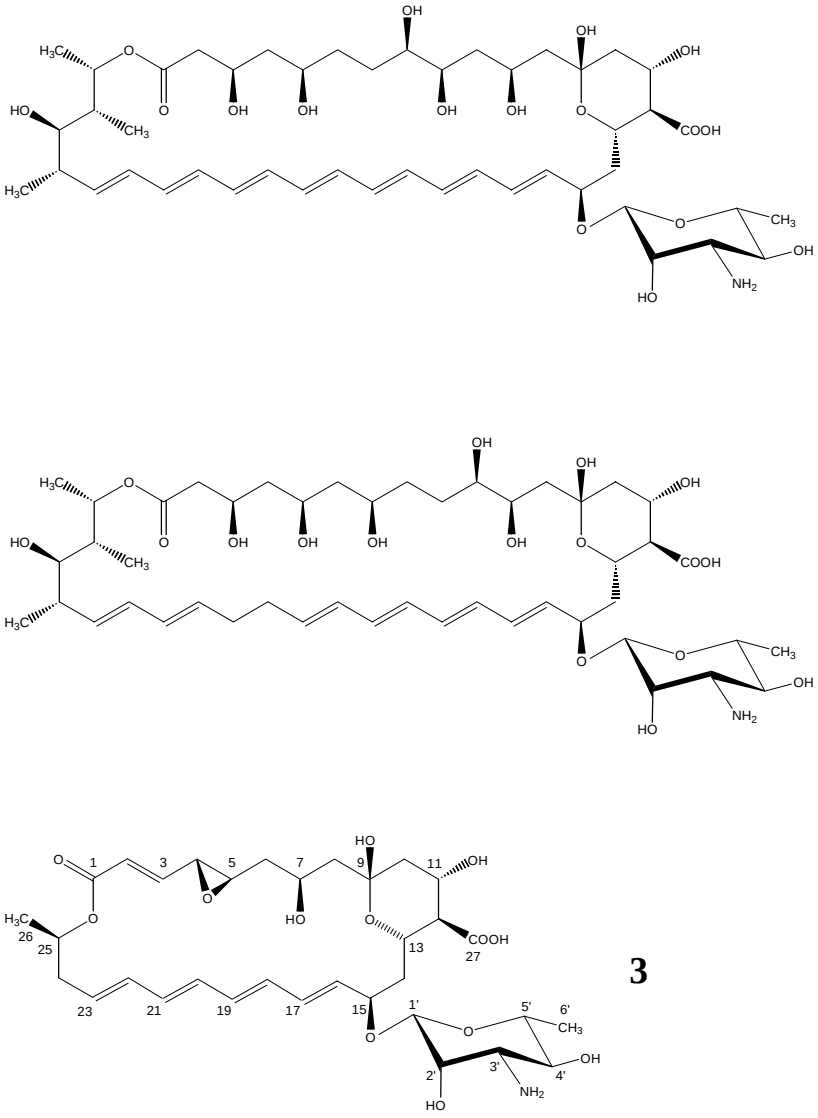

Figure 1. Chemical structures of polyene macrolide antibiotics. (**1**) amphotericin B, (**2**) nystatin A_1, and (**3**) natamycin.

Table 1. The United States Pharmacopeia and National Formulary (1999) designates the following descriptive terms for each range of relative solubilities.

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble, or Insoluble	10,000 and over

Table 2. Water solubility (mg/L) of natamycin as reported in literature.

mg/L	Reference	Year
50-100	Struyk et al.	1957
50-100	Clark et al.	1964
410	Marsh and Weiss	1967
52	Schaffner and Mechlinski	1972
50	Oostendorp	1981
30	Brik	1981

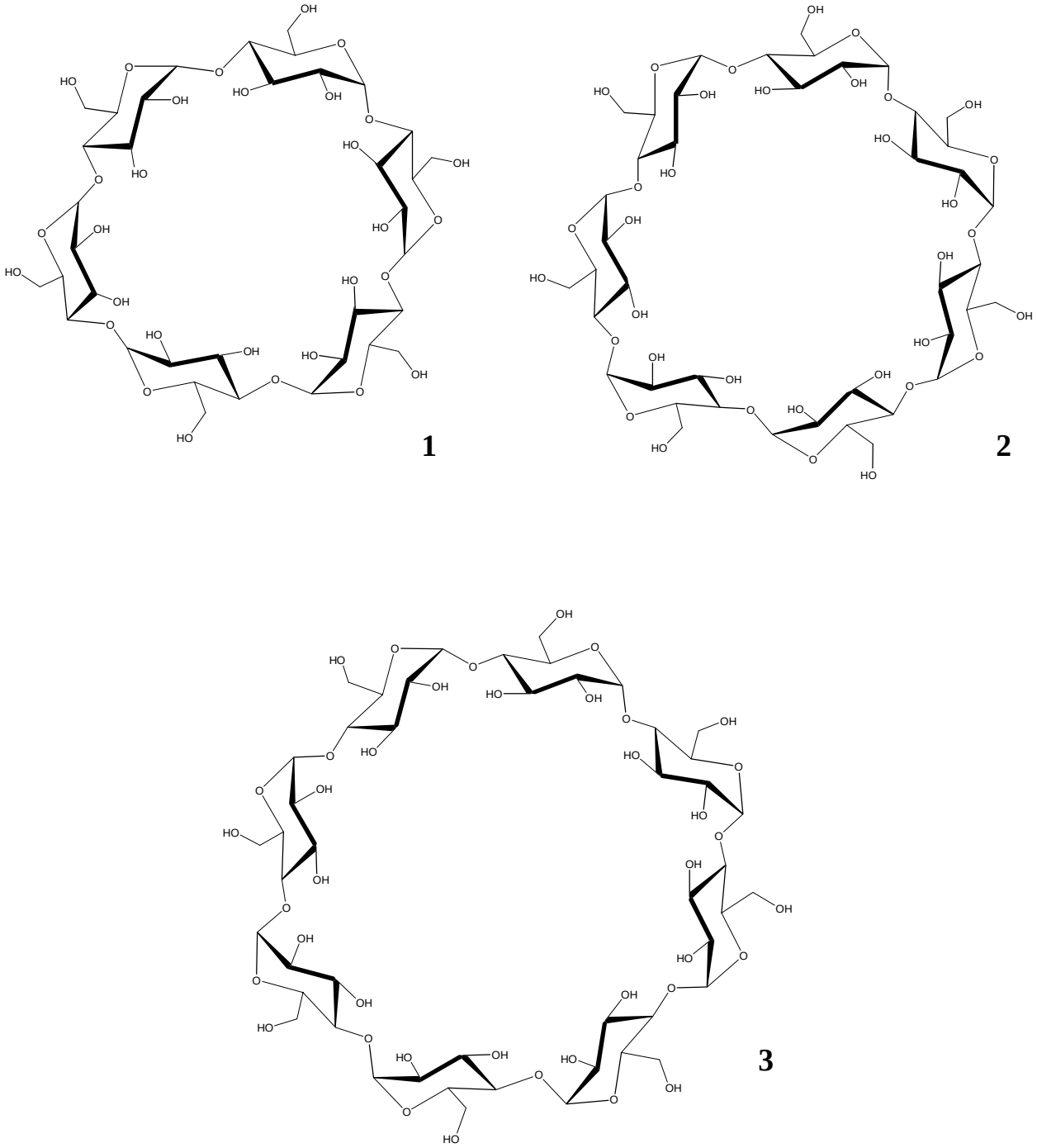

Figure 2. Chemical structures of the native cyclodextrins. (**1**) α-CD, (**2**) β-CD, and (**3**) γ-CD.

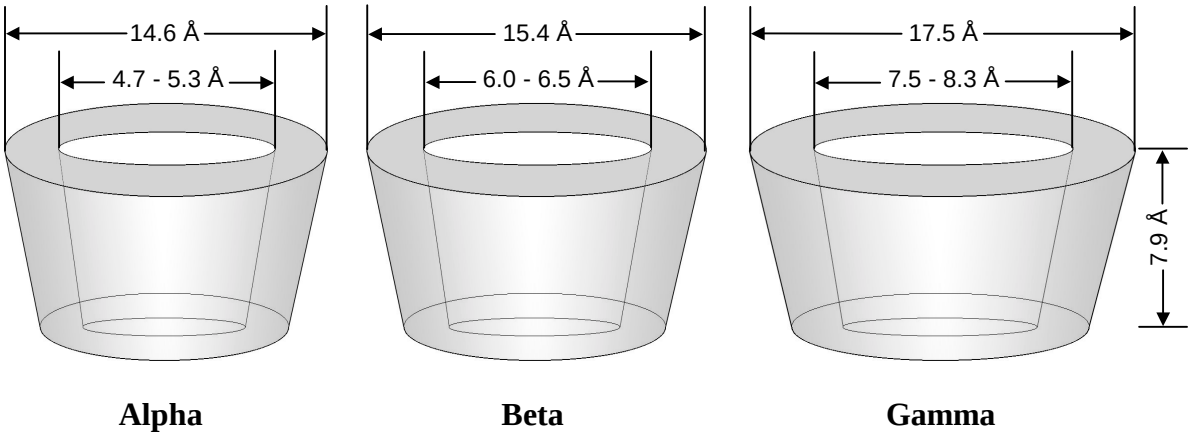

Figure 3. Molecular dimensions of the native cyclodextrins.

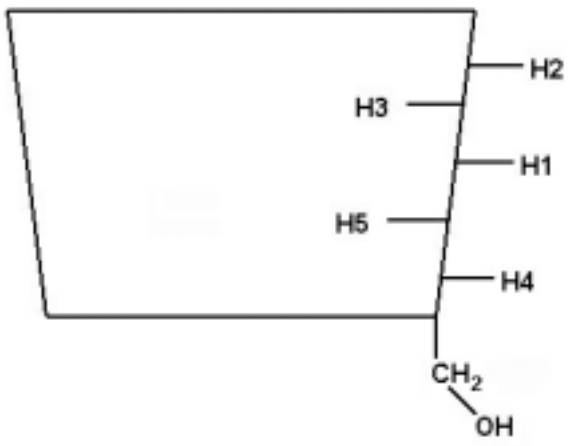

Figure 4. A two-dimensional depiction of the arrangement of the protons inside and outside the cyclodextrin cavity.

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