NEW HIGH-MOLECULAR DECOMPOSITION PRODUCTS OF NATAMYCIN* (PIMARICIN) WITH INTACT LACTONE-RING

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Mild acid treatment of natamycin (**IV**) results in biologically inactive aponatamycin (**VI**), an amphoteric substance with some natamycin-like chemical and physical properties. Aponatamycin contains one natamycin- and one natamycinolide-moiety. More drastic acid degradation of natamycin eliminates the aminosugar under formation of the dimer (**VII**) of the hypothetical aglycone of natamycin, natamycinolide (**V**) as well as a non-ionic compound, the dimer of the 12-decarboxy-11-anhydro analogue of natamycinolide.

In the past few years there has been an increasing interest in decomposition products of drugs and food additives, their structure, properties, and possible injurious side effects such as toxic or allergic reactions due to the decomposition products. This interest has centered on the antibiotics, most of which are more or less labile substances. Compared with most of the other groups of antibiotics very little is known about the decomposition products of the polyene macrolides, despite the existence of a large number of compounds belonging to this important group of antifungal agents.

Many authors have described degradation reactions of polyene antibiotics, using mainly chromatographic, spectrophotometric, and microbiological methods. However, the breakdown products were only seldom isolated. Altogether only a few decomposition products have been obtained in a more or less pure form. Mycosamine (**I**) is a major product of hydrolysis of the antimycotics amphotericin, nystatin, and natamycin (**IV**), three members of the important subgroup of the glycosidic polyenes. Its isolation as the hydrochloride from acid hydrolysed nystatin has been described by DUTCHER.[1] Furthermore, many polyenes are known to form long-chain heavily unsaturated aldehydes derived from the polyene portion of the macrolactone ring, by alkaline hydrolysis. Natamycin (**IV**), for example, yields[2,3] 13-hydroxy-2,4,6,8,10-tetradeca pentaen-1-al (**II**)**. Traces of this compound as well as greater quantities of mycosamine have been identified in pharmaceutical or industrial natamycin preparations (H. BRIK, unpublished data).

Decomposition products which still contain larger parts of the polyene molecule have been isolated after photolytical degradation of polyenes. The autoxidation is accompanied by the disappearance of a double bond at one end of the polyene chromophore (formation of an epoxide or a hydroperoxide). With this technique the tetraene nystatin has been transformed into a triene which was isolated as an amorphous white solid.[4] Autoxidation of aglyconic pentaenes like filipin and lagosin affords tetraene epoxides which have been isolated in a crystalline state.[5]

No degradation products with intact lactone-ring obtained by non-oxidative degradation of glycosidic polyenes have been described in the literature. Particularly attempts to obtain the hypothetical

* Generic name, approved by the WHO: natamycin (from *Streptomyces natalensis*). In earlier literature the name pimaricin has been used.

** Erroneously described by CEDER[3] as 13-hydroxy-2,4,6,8,10-dodecapentaen-1-al.

aglycones have failed. Removal of the aminosugar by acid hydrolysis of the glycosidic bond* always led to degradation or at least transformation of the polyene chromophore.

It was the purpose of our investigation to throw some light on the latter type of degradation, particularly the acid degradation of natamycin.

Results and Discussion

Acid Degradation Products of Natamycin

Degradation of natamycin in an acid medium produces, besides mycosamine, at least three inactive compounds which all have intact lactone-rings. Alcalimetric titration differentiated these three compounds into an amphoteric, an acidic, and a non-ionic substance.

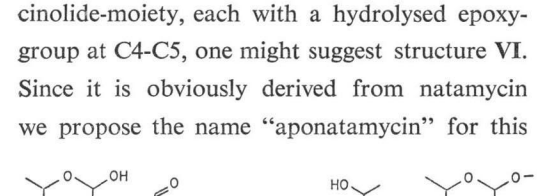
In anticipation of the complete elucidation of their chemical structures,[6] some evidence will be presented for the most remarkable property of these degradation products, *i.e.* their high molecular weight. Evidence for the molecular weight being high in comparison with that of the parent antibiotic was obtained from the following observations:

The dialysis rates are much lower than those of natamycin (m.w. 666) and nystatin**. They are of the same magnitude as that of candicidin, a polyene of the heptaene group with a m.w. of about 1,200. The volatility of the poly-trimethylsilyl derivatives was too low to obtain a useful mass spectrum. BOHLMANN[8] observed the same with the high-molecular polyene DJ-400 (m.w. 1,200~1,400).

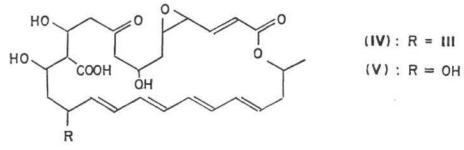
In contrast to natamycin and nystatin, dilute weakly alkaline solutions of the two water-soluble degradation products (the acidic and the amphoteric) as well as the polyene candicidin (m.w. about 1,200) give a precipitate with cationic surfactants. This reaction is given by many anionic high-molecular substances.[9]

Osmotic determination of the molecular weight gave an indication that each of these compounds contains two lactone rings. For the amphoteric compound, this agrees with the established presence of one basic to two acidic groups.

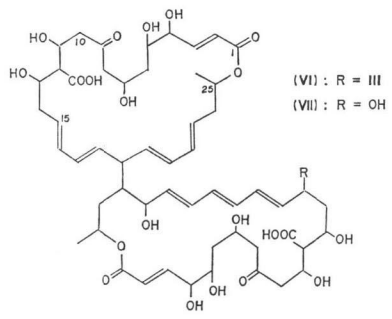
For the amphoteric compound which is probably built up from one natamycin- and one natamycinolide-moiety, each with a hydrolysed epoxy-group at C4-C5, one might suggest structure **VI**. Since it is obviously derived from natamycin we propose the name "aponatamycin" for this



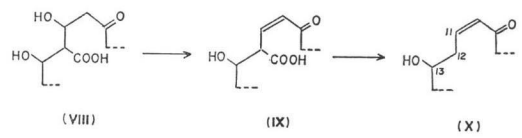
(I) (II) (III)



(IV) : R = III
(V) : R = OH



(VI) : R = III
(VII) : R = OH



(VIII) (IX) (X)

* Enzymatic attack of the glycosidic bond would be an interesting alternative.

** In contrast to our findings LAMPEN[7] reported that nystatin, in spite of its fairly low molecular weight of 926, is not dialysable due to the formation of micelles.

compound.

The acidic compound is formed by a chemical combination of two natamycinolide molecules and like aponatamycin it shows hydrolysed epoxy-groups (**VII**).

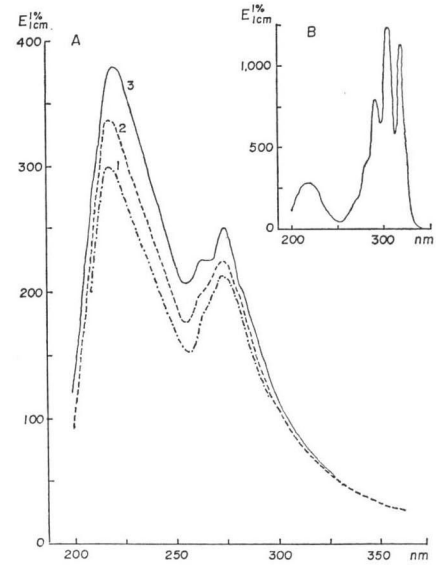
The non-ionic compound, in contrast to natamycin and the other degradation products, failed to generate carbon dioxide upon boiling in dilute sulphuric acid. Evidently decarboxylation has already taken place. Most probably it is the 12-decarboxy-11-anhydro-analogue of **VII** (partial structure **X**) formed by decarboxylation of the intermediate keto acid **IX**, itself resulting from β-elimination of the hydroxyl at C11 (**VIII**).

How exactly the dimerisation takes place is not yet quite clear. Structure **VI** represents one out of several possibilities, based on a coupling reaction between the two tetraene chains. This structure is consistent with ultraviolet spectral data (Fig. 1A).

Fig. 1. Ultraviolet spectra of methanolic solutions of:

A. Aponatamycin (1), di-natamycinolidediol (2), di-decarboxy-anhydronatamycinolidediol (3)

B. Natamycin



The ultraviolet absorption spectra of the three degradation products are qualitatively distinctly similar (Fig. 1A). The absorption at 215~220 nm, also on a molar basis, is identical to that of natamycin which is in conformity with the presence of an intact α,β-unsaturated lactone function. However, the strong absorption maxima of natamycin at 290, 303, and 318 nm, (Fig. 1B) characteristic for the all-*trans* tetraene structure, have completely disappeared. Instead a much lower and broader absorption at 274 nm is present which should probably be ascribed to a triene group. The abnormally low molar absorbtivity at 274 nm suggests a ratio of one chromophore to two macrolactone-units. The absorption which tapers off from the peak at 274 nm towards the visible region would be in agreement with the presence of several isomers, some of them containing *cis* diene structures.

Aponatamycin

Evidence for the existence of decomposition products of natamycin with an intact lactone ring was obtained for the first time, when an attempt was made to degrade natamycin at a moderately low pH. The degradation was executed in an aqueous suspension. At pH 1.5 a 5% w/v suspension of natamycin had lost its biological activity completely after having been kept in the dark for 2 months at room temperature or for 2 weeks at 40°C. From the reaction mixture aponatamycin was isolated as a light yellow amorphous substance in a rather high yield. The overall reaction may be presented by:

$$2\mathbf{IV}+2H_2O \longrightarrow \mathbf{VI}+\mathbf{I}\,.$$

Aponatamycin has an isoelectric point of about 3.5 (natamycin 6.5). This low value accounts for the very good solubility in water at pH 6~7 (> 20% w/v), compared with natamycin (at most 50 mcg/ml). Aponatamycin has also a very good solubility (> 20% w/v) in some strongly polar solvents and in a 1:1 mixture of methanol and chloroform. The low to very low solubility in certain water-

miscible solvents like alcohols and acetone is greatly enhanced by the addition of water. Natamycin is known to show a similar effect although in a much less pronounced way.

A curious fact is the strongly reduced solubility, even in dilute alkaline solution, of solid aponatamycin that has been kept for long periods (say a year) or has been brought to extreme dryness (a few hours, *in vacuo* over phosphorous pentoxide at 50°C).

Natamycinolidediol-dimer and its Decarboxyanhydro-analogue

When natamycin was degraded under more drastic conditions, *e.g.* in tetrahydrofuran - 2.5 N sulphuric acid* solution (2: 1) at reflux temperature for 2 hours or at room temperature for some days, the mycosamine moiety was totally cleaved from the molecule. When the solution was concentrated the remainder of the polyene molecule precipitated as a dark brown tarry mass. The tarry mass turned into a brown-yellowish powder when rubbed with water. By an extraction procedure the product could be segregated into an acidic and a neutral component, the di-natamycinolidediol and its decarboxy-anhydro-analogue, respectively. The following reaction-scheme illustrates the formations of these products:

$$2\mathbf{IV}+2H_2O \longrightarrow \mathbf{VII}+2\mathbf{I}$$
$$\mathbf{VII} \longrightarrow \mathbf{X}+2H_2O+2CO_2$$

The presence or absence of distinct ionic groups in these degradation products is reflected in the solubilities in solvents of different polarity. Whereas aponatamycin like natamycin itself is only soluble in some strongly polar solvents, di-natamycinolidediol dissolves also in higher alcohols and acetone. The decarboxylated compound dissolves readily in many solvents, but not in benzene or aliphatic hydrocarbons.

Comparison with the Acid Degradation of Nystatin

BOROWSKI[10] reported the formation of a pentaene chromophore from the tetraene portion of nystatin when this substance was hydrolysed in methanol-hydrochloric acid (the compound formed was not isolated). The new conjugated double bond was undeniably formed by elimination of mycosamine.

When we hydrolysed natamycin in the same way we could not detect any absorption maxima at wavelengths higher than 274 nm. In contrast, in the case of nystatin we were indeed able to observe a fairly high yield of a pentaene chromophore. With a view to the similarity in the parts of the macrolactone ring of nystatin and natamycin, that are responsible for this reaction, this may be called a surprising difference.

Experimental

General

Natamycin used for these studies was a production sample with a purity of 96%, calculated on a dry basis.

The degradation reactions were followed by thin-layer chromatography on precoated silica gel plates (Kieselgel 60 F254, Merck, Darmstadt, Germany) in a filter paper-lined chamber with chloroform - glacial acetic acid - methanol - water (6:2:2:1) as the developing solvent. The spots were detected by spraying first with ninhydrin solution (for mycosamine) and then, after heating for 10 minutes at 105°C, with conc. sulphuric acid. Rf-values: mycosamine 0.25; natamycin 0.4; aponatamycin 0.5; dinatamycinolidediol 0.7; decarboxyanhydro compound 0.85.

* With hydrochloric acid, addition of HCl to the epoxy-group takes place under formation of the chlorhydrin derivative.

Aponatamycin

A suspension of 50 g of natamycin in 1,000 ml of water was acidified to pH 1.5 with dilute hydrochloric acid and allowed to stand at 40°C for two weeks. The resulting ocher-yellow suspension was brought to pH 3.5 with dilute sodium hydroxide solution, coagulated by addition of ethyl ether, and filtered (37.4 g yellow powder).

To remove aglyconic byproducts the raw material was precipitated twice from methoxyethanol-ethyl ether and dried *in vacuo* at 40°C. Yield 17.1 g light-yellow amorphous powder. It darkens near 250°C but appears to be unaltered at temperatures up to 350°C*. $[\alpha]_D^{25} -18°$ (*c* 1, dimethylformamide). Equiv. weight theor. 611; found (by alcalimetric titration) about 595. Mol. weight theor. 1,222; found (by osmosis) 1,250 ± 100.

Di-natamycinolidediol and its Decarboxyanhydro-analogue

A quantity of 52 g of natamycin was dissolved in a mixture of 1,000 ml of tetrahydrofuran and 500 ml of 2.5 N sulphuric acid and allowed to stand for four days at room temperature. The green-brownish solution was concentrated *in vacuo* to remove the bulk of solvent. The resulting aqueous solution** was decanted from the brown tarry mass, after which the latter was solidified by rubbing with water several times. The resulting brown-yellowish powder was washed with water and dissolved in 500 ml of water by addition of dilute ammonia (pH 8). After addition of 25 g of sodium chloride (to prevent the formation of an emulsion) the solution was extracted three times with 200 ml of ethyl acetate. The aqueous layer was mixed with 200 ml of ethyl acetate and 200 ml of *n*-butanol and slowly acidified with dilute hydrochloric acid (pH 2) while stirring. The upper layer was washed and dried with anhydrous sodium sulphate. The solution was concentrated *in vacuo* to a volume of about 100 ml and poured slowly into 1,000 ml of benzene. The precipitate was filtered, washed and dried. Yield 24 g dinatamycinolidediol as a light-yellow amorphous powder. It darkens near 200°C and appears to be unaltered at temperatures up to 350°C. $[\alpha]_D^{25} -6°$ (*c* 1, dimethylformamide). Equiv. weight, theor. 538.5; found (by alcalimetric titration) about 530. Mol. weight, theor. 1077; found (by osmosis) 1,030 ± 80.

The combined ethyl acetate extracts were washed with 0.1 N sodium hydroxide solution and concentrated to dryness *in vacuo*. The residue was stirred thoroughly with 20 ml of ethyl acetate and filtered. The filtrate was poured into 50 ml of benzene, the precipitate was filtered and washed with benzene. Yield 0.52 g of cream-coloured di-decarboxyanhydronatamycinolidediol. The product darkens near 130°C and is slightly sintered at 300~350°C. $[\alpha]_D^{25} -5°$ (*c* 1, dimethylformamide). M.w., theor. 917; found (by osmosis) 975 ± 75.

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* Natamycin sinters at 250°C but does not melt below 350°C.

** When hydrochloric acid is used instead of sulphuric acid it is possible to isolate mycosamine hydrochloride from the remaining solution in a very simple way. The latter was decolourized with charcoal and concentrated to dryness *in vacuo*. Yield 12.4 g (80% of theory).

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