Inhibitory Effect of Modified Bafilomycins and Concanamycins on P- and V-Type Adenosinetriphosphatases†

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ABSTRACT: Various ATPases have been tested for their sensitivity to naturally occurring unusual macrolides and their chemically modified derivatives, which are structurally related to bafilomycin A_1 (**1**), the first specific inhibitor of vacuolar ATPases. The structure–activity study showed that in general the concanamycins, 18-membered macrolides, are better and more specific inhibitors than the bafilomycins of this class of membrane-bound ATPases. The additional carbohydrate residue is not responsible for the improved activity. The importance of an intact hemiketal ring, which is part of an intramolecular hydrogen-bonding network, and the effects of the size of the macrolactone ring are discussed. The structurally related elaiophylin (**13**), a C_2-symmetric macrodiolide antibiotic, proved to be inactive on vacuolar ATPases but still retained its inhibitory effect on P-type ATPases.

The bafilomycins (Figure 1), unusual macrolide antibiotics with a 16-membered lactone ring, were isolated from *Streptomyces griseus* (Werner et al., 1984). These compounds inhibited growth of Gram-positive bacteria and fungi in a disc diffusion assay. In addition, bafilomycin C_1 (L-681,110 A_1 in earlier work) was reported to inhibit the enzymatic activity of the Na^+,K^+-ATPase (Hensens et al., 1983; Huang et al., 1984). More recently, in a detailed analysis we compared the effects of bafilomycin A_1 (**1**)[1] on representative enzymes of the three classes of ATPases (Bowman et al., 1988). The results revealed that the F_1F_o ATPases (F-type) from bacteria and mitochondria are not affected by this antibiotic. In contrast, E_1E_2 ATPases (P-type), e.g., the K^+-dependent ATPase (Kdp-ATPase) from *Escherichia coli*, the Na^+,K^+-ATPase from ox brain, and the Ca^{2+}-ATPase from sarcoplasmic reticulum, are moderately sensitive to this inhibitor. However, membrane ATPases (V-type) from *Neurospora* vacuoles, chromaffin granules, and plant vacuoles are extremely sensitive. In addition, calculations showed it to be highly probable that bafilomycin A_1 (**1**) interacts stoichiometrically with V-type ATPases. For the first time these results showed convincingly that bafilomycin A_1 (**1**) is useful for distinguishing among the different types of ATPases and that it is a potent, relatively specific inhibitor of the vacuolar ATPases (Bowman et al., 1988). Because of its specificity, bafilomycin A_1 has been important in the identification of V-type ATPases in various organelles and has stimulated research on the physiological roles of these ATPases (Sundquist et al., 1990; Moriyama & Nelson, 1989; Mattsson et al., 1991; Umata et al., 1990; Yoshimori et al., 1991; Martinez-Zaguilán et al., 1992). In vitro, it appears that bafilomycin A_1 (**1**) exerts its inhibitory effect by tight binding to the enzyme complex (Hanada et al., 1990) rather than by covalent modification. In contrast, in vivo studies revealed that the effect of bafilomycin A_1 (**1**) is reversible (Yoshimori et al., 1991). However, the possibility that the loss of the inhibitory effect is due to de novo synthesis of the vacuolar ATPase has not been ruled out.

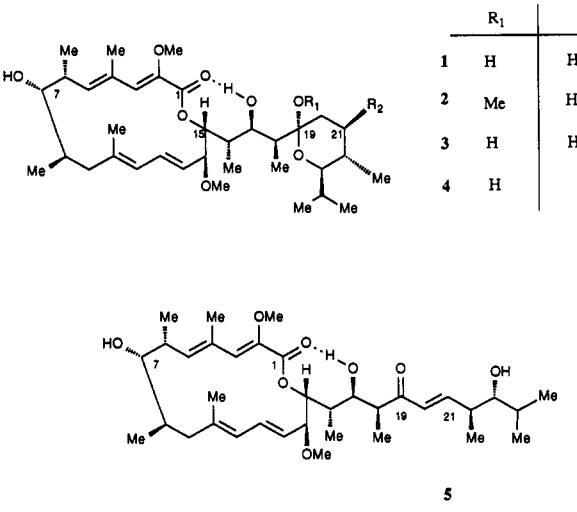

FIGURE 1: Structure of bafilomycins and bafilomycin derivatives.

The concanamycins (Figure 2), another family of unusual macrolide antibiotics, were isolated first from *Streptomyces diastatochromogenes* (Kinashi et al., 1984) and recently from unidentified *Streptomyces* sp. (Woo et al., 1992a; Bindseil & Zeeck, 1993). They are effective inhibitors of the proliferation of mouse splenic lymphocytes, which is stimulated by concanavalin A, and they are biologically active against several fungi and yeasts but not against bacteria (Kinashi et al., 1984). They have also been shown to exhibit immunosuppressive activity (Vanek et al., 1991) and to inhibit acidification of lysosomes (Woo et al., 1992b). The concanamycins (Figure 2), which are structurally strongly related to the bafilomycins (Figure 1), possess an 18-membered lactone ring and a 6-membered hemiketal ring in which 23-OH is glycosylated

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[1] The bold numbers refer to the numbering of the macrolide antibiotics given in Figures 1–3.

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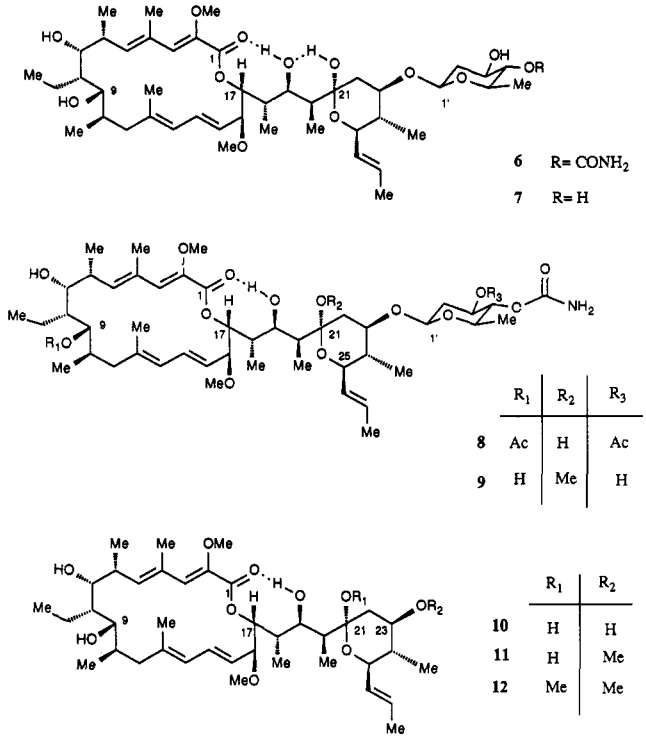

FIGURE 2: Structure of concanamycins and concanamycin derivatives.

by 2-deoxy-β-D-rhamnose (Kinashi et al., 1984). The stereochemistry of comparable centers of the bafilomycins and concanamycins is identical, and the conformation of both macrolide families is very similar because of an identical hydrogen-bonding system (Westley et al., 1984; Baker et al., 1989).

In this paper, we tested the effects of the concanamycins on representative enzymes of the three classes of ATPases in comparison to bafilomycin A_1. In addition, we have tested the inhibitory effects of modified bafilomycins and concanamycins with the aim of probing structure–activity relationships and of developing a strategy for synthesizing modified antibiotics able to bind covalently to the enzyme complex.

MATERIALS AND METHODS

Preparative Procedures. Kdp-ATPase from *E. coli* strain TKA1000 was prepared as described by Siebers et al. (1992), using the two-column protocol. The ATP synthase from *E. coli* KY7485 was prepared as described (Schneider & Altendorf, 1986; Friedl & Schairer, 1986). Vacuolar membranes of *Neurospora crassa* were prepared as described (Bowman & Bowman, 1988).

Assays. The K^+-stimulated ATPase activity of the Kdp complex was measured as described previously (Siebers et al., 1988). The vacuolar ATPase activity of *N. crassa* was measured as detailed in Bowman and Bowman (1988). The assay for the ATPase activity of the *E. coli* F_1F_o was performed as described previously (Deckers-Hebestreit et al., 1992). Before the reaction was started by the addition of ATP, the samples were preincubated with the corresponding antibiotic for 1 min at 37 °C.

Stock solutions of the macrolide antibiotics were dissolved in dimethyl sulfoxide and stored at −20 °C. The actual concentrations of stock solutions were determined spectrophotometrically by using the molar extinction coefficients shown in Table I. Control samples without macrolide antibiotics contained dimethyl sulfoxide; the final concentrations of dimethyl sulfoxide in the assay mixtures did not exceed 0.1%. The macrolides were present in the assay mixtures at the concentrations given in the figures.

Table I: Molar Extinction Coefficients of Macrolide Antibiotics

macrolide antibiotic	molar extinction coeff (M^{-1} cm^{-1})
bafilomycin A_1 (**1**)	ϵ_{245}: 32 000[a]
bafilomycin A_2 (**2**)	ϵ_{245}: 32 000[a]
21-*O*-acetylbafilomycin A_1 (**3**)	ϵ_{245}: 32 000
bafilomycin A_1 21-ketone (**4**)	ϵ_{247}: 42 000
bafilomycin D (**5**)	ϵ_{242}: 29 000[b]
concanamycin A (**6**)	ϵ_{245}: 40 500[c]
concanamycin C (**7**)	ϵ_{245}: 40 800[d]
3′,9-di-*O*-acetylconcanamycin A (**8**)	ϵ_{244}: 30 000
21-*O*-methylconcanamycin A (**9**)	ϵ_{244}: 36 000
concanolide (**10**)	ϵ_{245}: 31 500
23-*O*-methylconcanolide (**11**)	ϵ_{245}: 40 000
21,23-di-*O*-methylconcanolide (**12**)	ϵ_{245}: 41 000
elaiophylin (**13**)	ϵ_{253}: 67 500[e]

[a] In contrast to 25 000 reported for **1** and **2** in the original work (Werner et al., 1984). [b] Kretschmer et al., 1985. [c] Kinashi et al., 1981. [d] Kinashi et al., 1982. [e] Kaiser et al., 1981.

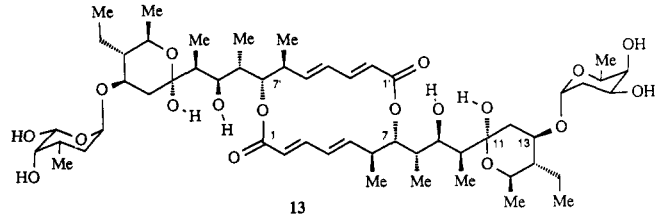

FIGURE 3: Structure of elaiophylin.

Protein concentrations were determined by the method of Dulley and Grieve (1975) or of Lowry et al. (1951) or, in detergent-containing samples, with the Pierce–BCA (bicinchoninic acid) protein assay reagent (Smith et al., 1985), following the instructions of the manufacturer. Bovine serum albumin was used as protein standard throughout.

Antibiotics. Concanamycins A (**6**) and C (**7**) were isolated from the fermentation broth of *Streptomyces* sp. (strain Gö 22/15). Bafilomycins A_1 (**1**) and A_2 (**2**) were isolated from the fermentation broth of *Streptomyces griseus* (strain 2599). The four antibiotics were purified as described (Bindseil & Zeeck, 1993). Concanamycin A contained up to 25% of the 8-deethyl-8-methyl homologue, concanamycin B, which could not readily be removed by chromatography. Pure elaiophylin (**13**) was a gift of M. Gerlitz and J. Rohr (Universität Göttingen). The new derivatives mentioned in this paper were prepared as described by Bindseil and Zeeck (1992). The derivatives of concanamycin A (**6**) contained small amounts of their 8-deethyl-8-methyl homologues, while the concanolide derivatives were prepared from pure concanamycin C (**7**) and were free of homologues. The molecular extinction coefficients of the macrolides are given in Table I. The complete structural formulas, which were proven by spectroscopic methods, especially 2D-NMR spectroscopy, are given in Figures 1–3.

RESULTS

Bafilomycin A_1 (**1**) has proved to be an excellent inhibitor (K_i in the nanomolar range) of V-type ATPases. Consequently, the need for that compound has risen tremendously during the last few years. However, the isolation of the antibiotic is a multistep process, and the compound has proved to be rather unstable. In search of other more stable compounds, which have similar effects on the various ATPases but are easier to purify, we have tested concanamycin A (**6**).

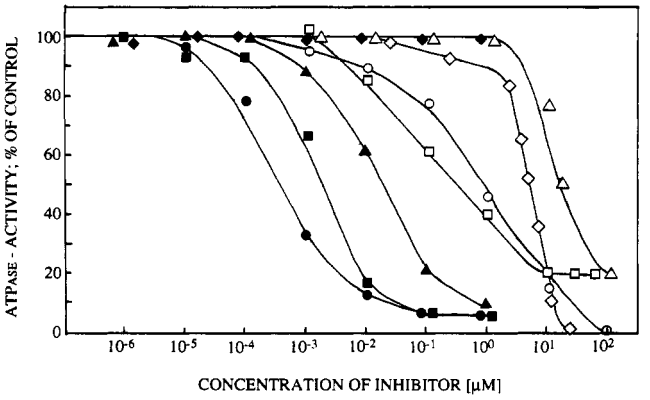

FIGURE 4: Inhibitory effects of bafilomycin A_1 (●, ○), 21-*O*-acetylbafilomycin A_1 (■, □), bafilomycin D (▲, △), and elaiophylin (◆, ◇) on the activity of the Kdp-ATPase from *E. coli* (open symbols) and the vacuolar ATPase from *N. crassa* (closed symbols). Assays were performed as described, using 36 μg of the Kdp-ATPase in an assay volume of 10 mL or 5 μg of the vacuolar ATPase in an assay volume of 0.5 mL. The 100% values of specific ATPase activity were 0.8 μmol mg^{-1} min^{-1} for the Kdp-ATPase and 1.2 μmol mg^{-1} min^{-1} for the *N. crassa* vacuolar ATPase, respectively.

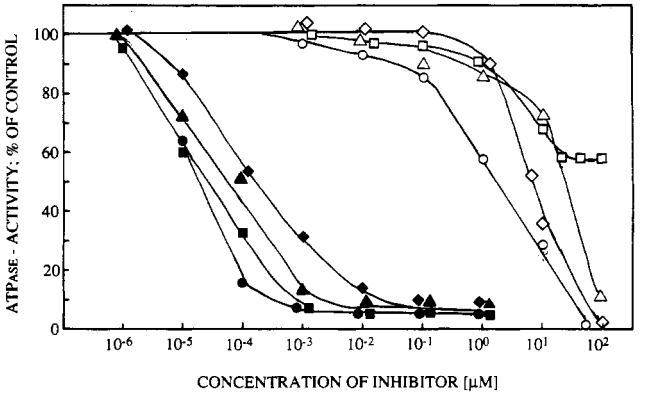

FIGURE 5: Inhibitory effects of concanamycin A (●, ○), concanamycin C (■, □), concanolide (▲, △), and 21-*O*-methylconcanamycin A (◆, ◇) on the activity of the Kdp-ATPase from *E. coli* (open symbols) and the vacuolar ATPase from *N. crassa* (closed symbols). Assay conditions and the 100% values of specific ATPase activities were as described in the caption to Figure 4.

Measurements with the three different classes of ATPases revealed that the ATP synthase (F-type) from *E. coli* was not affected at concentrations of **6** up to 50 μM (equivalent to 150 μmol of **6**/mg of protein; data not shown). By contrast, the Kdp-ATPase (P-type) showed intermediate sensitivity (Figure 5), with an I_{50} value of 0.5 μmol mg^{-1} (Table II). The most striking result, however, was found with the vacuolar ATPase (V-type) from *N. crassa* (Figure 5; Table II): the membrane-bound ATPase exhibited high sensitivity to this antibiotic, with an I_{50} value of 0.002×10^{-3} μmol mg^{-1}. Thus, concanamycin A (**6**), compared to bafilomycin A_1 (**1**), shows an even greater specificity for the different kinds of ATPases because of its more than 20 times improved inhibitory effect on the V-type ATPase.

In comparison to **6**, the carbamoyl group attached to the sugar hydroxy group at position C-4′ is missing in concanamycin C (**7**) (Figure 2). This modified compound shows a very weak inhibitory effect on the P-type ATPase (Table II). However, due to solubility problems of **7** in water, only concentrations up to 10–20 μM could be tested (Figure 5). In contrast, concanamycin C (**7**) has retained its specific inhibitory effect on the V-type ATPase (Figure 5; Table II).

3′,9-Di-*O*-acetylconcanamycin A (**8**) was as potent as the parent compound **6** on the V-type ATPase, but 21-*O*-methylconcanamycin A (**9**) had reduced activity.

In concanolide (**10**), the chemically derived aglycon of the concanamycins, the sugar residue is absent, moving this derivative structurally closer to bafilomycin A_1 (**1**). In comparison to **6**, the inhibitory action toward P- and V-type ATPases is reduced by a factor of 3–4 (Table II), but the inhibition of the V-type ATPase remains nearly 10-fold better than for bafilomycin A_1 (**1**). If, in addition to the missing rhamnosyl group, the positions C-21 and C-23 of concanolide are methylated as in **11** or **12** (Figure 2), the inhibitory effect toward the V-type ATPase is reduced drastically; e.g., 21,-23-di-*O*-methylconcanolide (**12**) retained less than 1/100 the activity of **6** (Table II). Similar but not as drastic losses of activity can be observed for the bafilomycin A_1 derivatives (Figure 1) bafilomycin A_2 (**2**), 21-*O*-acetylbafilomycin A_1 (**3**), and bafilomycin A_1 21-ketone (**4**). Because the acetylated compounds are less soluble in water, measurements with concentrations above 5–10 μM with the P-type ATPase were difficult (Figure 4). The assay mixture turned turbid, and no further inhibition could be observed. However, total inhibition could still be achieved by addition of bafilomycin A_1 (data not shown). In bafilomycin D (**5**) the six-membered hemiketal ring has been opened (Figure 1). This modification has a pronounced effect on the inhibitory action on both P- and V-type ATPases. Both enzymes, compared to **1**, are more than 20-fold less sensitive toward **5** (Figure 4; Table II).

A structurally and functionally interesting unusual macrodiolide antibiotic with C_2 symmetry is elaiophylin (**13**) (Figure 3) (Fiedler et al., 1981; Kaiser et al., 1981), which contains structural elements of both bafilomycin A_1 (**1**) and concanamycin A (**6**). Elaiophylin (**13**) was isolated from different *Streptomyces* sp. and exhibits antibacterial as well as in vivo anthelminthic activity (Gerlitz et al., 1992). Since the hydrophobic part of the macrolide ring has been eliminated and the hemiketal ring, substituted with a 2-deoxy-β-D-rhamnosyl moiety, has been added twice, at positions C-7 and C-7′ of the macrodiolide ring, **13** is much more hydrophilic than bafilomycin A_1 (**1**) and concanamycin A (**6**). Compared to **6**, this antibiotic is a weaker inhibitor for the P-type ATPase, whereas it does not affect the V-type ATPase at all (Figure 4; Table II).

DISCUSSION

Inhibitors have played an important role in studying ion-translocating ATPases. Specific inhibitors, such as vanadate and ouabain for P-type ATPases and azide and oligomycin for F-type ATPases, have served both to distinguish among groups of ATPases and to identify new ATPases, including the vacuolar group (Bowman & Bowman, 1986; Mellman et al., 1986; Sze, 1985). Inhibitors have also been valuable in analyzing the strucure and mechanism of ATPases. For example, vanadate, which is a transition-state analogue of phosphate, provided a means to demonstrate the formation of a phosphorylated intermediate of the enzyme reaction cycle of P-type ATPases (Cantley et al., 1978), while *N,N′*-dicyclohexylcarbodiimide has been used in numerous studies to investigate proton translocation by ATPases [e.g., see Sussmann and Slayman (1983), Fillingame (1980), and Sun et al. (1987)]. Finally, inhibitors that include *N,N′*-dicyclohexylcarbodiimide (Fillingame, 1975), oligomycin (Edwards & Unger, 1978), and hygromycin (McCusker et al., 1987) have provided a means for obtaining mutants of membrane ATPases. Recently, we have identified the first inhibitor, bafilomycin A_1 (**1**), which is highly specific for V-type ATPases (Bowman et al., 1988).

Table II: Sensitivity of Two ATPases to Macrolide Antibiotics

macrolide antibiotic	H^+-ATPase (*N. crassa*)		Kdp-ATPase (*E. coli*)	
	K_i (nM)	I_{50} (μmol mg^{-1})	K_i (μM)	I_{50} (μmol mg^{-1})
bafilomycin A_1 (**1**)	0.5	0.05×10^{-3}	1.3	0.36
bafilomycin A_2 (**2**)	1.0	0.1×10^{-3}		
21-*O*-acetylbafilomycin A_1 (**3**)	1.3	0.13×10^{-3}	1.2[a]	0.33[a]
bafilomycin A_1 21-ketone (**4**)			1.7	0.47
bafilomycin D (**5**)	20.0	2×10^{-3}	20.8	5.64
concanamycin A (**6**)	0.02	0.002×10^{-3}	1.8	0.5
concanamycin C (**7**)	0.02	0.002×10^{-3}	*b*	*b*
3′,9-di-*O*-acetylconcanamycin A (**8**)	0.02	0.002×10^{-3}	3.3[a]	0.91[a]
21-*O*-methylconcanamycin A (**9**)	0.2	0.02×10^{-3}	5.6	1.54
concanolide (**10**)	0.06	0.006×10^{-3}	8.4	2.33
23-*O*-methylconcanolide (**11**)			4.7[a]	1.3[a]
21,23-di-*O*-methylconcanolide (**12**)	3.0	0.3×10^{-3}	7.1[a]	1.97[a]
elaiophylin (**13**)	ni[c]	ni[c]	5.0	1.39

[a] Due to solubility problems, 100% inhibition could not be achieved. [b] Due to solubility problems, only 30–40% inhibition could be obtained, making the determination of the K_i and I_{50} values impossible. [c] No inhibition.

In this paper, we identify concanamycin A (**6**) as another valuable tool for distinguishing among the three different types of ATPases. In comparison to **1**, the vacuolar ATPase proved to be more sensitive to **6**; the P-type ATPase was moderately affected by this antibiotic, whereas the F-type ATPase was not impaired at all. This almost identical inhibitory pattern between bafilomycin A_1 and concanamycin A is based on structural and conformational similarities between the two compounds (Figures 1 and 2). The increase in size of the macrolide ring and the additional rhamnosyl group resulted in improved inhibitory properties of concanamycin A (**6**) compared with bafilomycin A_1 (**1**) toward the vacuolar ATPase. This effect is maintained in concanolide (**10**), the aglycon of **6**. Therefore, the carbohydrate residue, although it is essential for the greater stability of **6**, probably does not play an important role in the inhibitory mechanism. This is in accordance with the observation that the removal of the carbamoyl group at position C-4′ of the sugar in concanamycin C (**7**) does not change the effect of this compound on the V-type ATPase. The effect of the carbamoyl group on the P-type ATPase is more difficult to evaluate since in comparison to **6** the solubility of **7** in H_2O has been reduced. From the structural point of view, this reduced solubility is difficult to rationalize.

The hemiketal hydroxy group is thought to contribute to the conformational stability of the macrolide antibiotics and to be essential for their biological activities (Baker et al., 1989). Methylation as in **2**, **9**, **11**, and **12** should influence this rigid conformation, yet exerts only a minor effect on the inhibitory characteristics of the derivatives **9**, **11**, and **12** toward the Kdp-ATPase. However, methylation as in **9** and **12** had a more severe effect on the inhibitory characteristics toward the V-type ATPase. The latter is in accord with the observation that the methylated derivatives **9**, **11**, and **12** showed markedly reduced biological activities against fungi (Bindseil and Zeeck, data not shown).

The hemiketal ring itself seems to play an important role in biological activity, since bafilomycin D (**5**) is 20 times less active than bafilomycin A_1 (**1**) against both P- and V-type ATPases and 1000 times less active than concanamycin A (**6**) against the V-type ATPase. This would lead to the conclusion that the macrolide ring plays a key role in biological activities. In this context it is noteworthy that the increased size of the macrolide ring in the concanamycins compared to the bafilomycins resulted in a greater conformational flexibility in the hydrophobic part of these compounds (Kinashi et al., 1984; K. U. Bindseil and A. Zeeck, manuscript in preparation). This flexibility might be the reason for the increased inhibitory activities of **6** and **7** and of some of their derivatives on V-type ATPases. However, elaiophylin (**13**), which possesses a 16-membered macrodiolide ring, two slightly modified hemiketal rings, and two hydrogen bond systems, is only a weak inhibitor for P-type ATPases and has no effect on V-type ATPases. The latter observation can be explained by the drastically changed molecular geometry and the fact that **13** is more hydrophilic and, therefore, may have problems in reaching its natural binding site.

A similar conclusion that the 18-membered macrolide ring and the 6-membered hemiketal ring of concanamycins are responsible for the potent inhibitory effect on lysosomal acidification in rat liver has been reached by Woo et al. (1992a). Furthermore, inhibition of the acidification of endosomes and lysosomes by concanamycin B in macrophage J774 has also been reported (Woo et al., 1992b). However, in these cases the effect of the various compounds on the activity of the V-type ATPases, which may be responsible for the observed acidification, has not been tested.

Besides some circumstantial evidence that bafilomycin A_1 (**1**) binds to the V_o-part of the V-type ATPases (Bowman and Bowman, unpublished observation; Hanada et al., 1990), the actual binding site of the antibiotic within the P- and V-type ATPases is unknown. In order to obtain information about that, the synthesis of modified compounds (e.g., nitrene- and carbene-generating derivatives) able to form a covalent bond with the enzyme complex while retaining their specific inhibitory properties should be rewarding. Therefore, we have set out to introduce modifications at position C-21 of bafilomycin A_1 (**1**). Since acetylation or even oxidation of the hydroxy group has no significant effect on the inhibitory properties of these modified compounds, this position seems to be a promising site for further chemical modifications. Since concanamycin A (**6**) is a more stable compound than bafilomycin A_1 (**1**), the former might be more suited for further chemical modifications.

We conclude that as specific inhibitors of high potency, bafilomycin A_1 (**1**) and concanamycin A (**6**) and their relatives appear to be excellent candidates for probing the structure and function of the V-type ATPases. The bafilomycins and concanamycins also show promise for probing the structure of the Kdp-ATPase and may prove useful for other P-type ATPases as well.

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