Semisynthetic Derivatives of Concanamycin A and C, as Inhibitors of V- and P-Type ATPases: Structure−Activity Investigations and Developments of Photoaffinity Probes†

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ABSTRACT: V-type ATPases are inhibited by the plecomacrolides bafilomycin and concanamycin, which exert their inhibitory potential at nanomolar concentrations. In addition, some P-type ATPases are inhibited at micromolar concentrations. We initiated intensive structure−activity investigations with semisynthetic concanamycin derivatives to approach the following two questions: (i) What is the pharmacophor, the structural key element, of the plecomacrolides that leads to their inhibitory potential against V- and P-type ATPases? (ii) Where is the binding site within these two different types of ATPases? In a first step, we examined where chemical modifications (O-acylations, substitutions, eliminations) could be placed without seriously affecting the inhibitory potential of the macrolides. In a second step, we used the knowledge of these structure−activity investigations to introduce traceable elements (fluorescent or radioactive) or nitrene-generating azido or carbene-generating diazirine-groups able to bind the inhibitors to their target covalently. These studies led finally to the synthesis of two photoaffinity probes that were used in labeling experiments with the purified plasma membrane V-type ATPase of *Manduca sexta* (described in a following paper, Huss, M., Gassel, M., Ingenhorst, G., Dröse, S., Zeeck, A., Altendorf, K., Wieczorek, H., manuscript submitted).

Concanamycins and bafilomycins are the most prominent members of the plecomacrolides (overview in ref *1*). These secondary metabolites are produced by different *Streptomyces* species during mycelia generation (*2*−*5*). While these closely related "unusual macrolides" showed various "antibiotic effects" in early screening studies (overview in ref *6*), their mode of action remained elusive until empirical studies with different ion-translocating ATPases had been carried out (*7*, *8*). In these studies, their potential as V-type ATPase inhibitors with high affinity (inhibition occurs in vitro and in vivo at nanomolar concentrations) was discovered. In addition, it became clear that the related F-type ATPases (ATP synthases) were not affected by these macrolide antibiotics, whereas some P-type ATPases, the third large ATPase-group included in these studies, were inhibited at micromolar concentrations (*7*, *8*). Bafilomycin A_1 [**1**][1] and concanamycin A [**3**] (Figure 1) are now commonly used in studies of the physiological roles of V-type ATPases in cell cultures and in cellular processes that require the activity of these V-type ATPases, such as receptor-mediated endocytosis, toxin and virus invasion into eukaryotic cells, protein sorting, etc. (references are given in ref *6*). Moreover, the involvement of V-type ATPases in processes such as bone resorption, where a malfunction can cause osteoporosis (*9*), and in the invasion of several bacterial toxins (*10*−*14*) and some viruses (*15*−*17*) turned the plecomacrolides into interesting compounds for pharmacological studies (*18*−*20*). However, the almost indistinguishable inhibition of V-type ATPases from different organisms and tissues has thus far

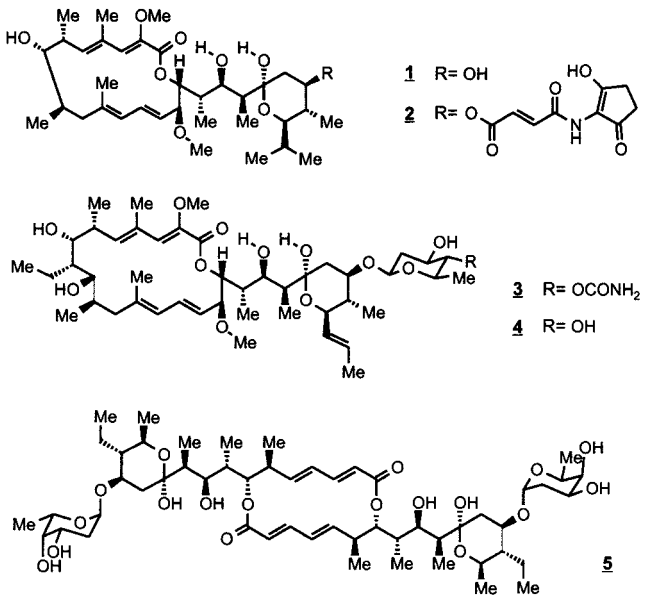

FIGURE 1: Structures of the bafilomycins [**1** and **2**], concanamycins [**3** and **4**], and elaiophylin [**5**].

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[1] The bold numbers refer to the numbering of the macrolide antibiotics given in Figures 1−4 and Table 1.

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Table 1: Extinction Coefficient of the Plecomacrolide Derivatives

plecomacrolide	ϵ^{MeOH} [M^{-1} cm^{-1}]	ref
bafilomycin A_1 [**1**]	ϵ_{245}: 32 000	*8*
bafilomycin B_1 [**2**]	ϵ_{248}: 35 000	*4*
concanamycin A [**3**]	ϵ_{245}: 40 500	*62*
concanamycin C [**4**]	ϵ_{245}: 40 800	*63*
elaiophylin [**5**]	ϵ_{253}: 67 500	*61*
3*R*,4*S*,5*R*-3-*O*-(β-D-2-deoxyrhamnopyranosyl)-4-methyl-6-octenic acid δ-lactone (prelactone C-glycoside) [**6**]	endabsorption	*42*
3*R*,4*S*,5*R*-3-hydroxy-4-methyl-6-octenic acid δ-lactone (prelactone C) [**7**]	endabsorption	*34*
4*R*,5*S*,6*R*-3-*O*-(α-L-deoxyfucopyranosyl)-4-ethyl-hexanoic acid δ-lactone (prelactone E-glycoside) [**8**]	endabsorption	*42*
21-deoxyconcanamycin A [**9**]	ϵ_{245}: 35 000	*60*
21-deoxyconcanolide A [**10**]	ϵ_{245}: 39 000	*41*
23-*O*-benzoyl-21-deoxyconcanolide A [**11**]	ϵ_{237}: 40 000	*41*
9-*O*-benzoyl-21-deoxyconcanolide A [**12**]	ϵ_{235}: 44 900	*41*
9-*O*-oleoyl-21-deoxyconcanamycin A [**13**]	ϵ_{245}: 41 400	*41*
3′-*O*-(4-azidobenzoyl)-concanamycin C [**14**]	ϵ_{247}: 50 000	*41*
3′,4′-di-*O*-(4-azidobenzoyl)-concanamycin C [**15**]	ϵ_{248}: 49 400	*41*
3′-*O*-(9-anthracenoyl)-concanamycin C [**16**]	ϵ_{252}: 180 100	*41*
9-*O*-([3,5-^{3}H]-4-azidobenzoyl)-21-deoxy−concanamycin A [**17**]	nd	*41*
3′-*O*-(9-anthracenoyl)-4′,9-di-*O*-(4-azidobenzoyl)-concanamycin C [**18**]	ϵ_{252}: 123 900	*41*
3′-*O*-[3-(anthracen-9-yl)-propionoyl]-9-*O*-(4-azidobenzoyl)-concanamycin A [**19**]	ϵ_{255}: 161 800	*41*
3′-*O*-[3-(anthracen-9-yl)-propionoyl]-9-*O*-acetyl-21-(4-azidobenzoylperoxy)-concanamycin A [**20**]	nd	*41*
16-demethyl-21-deoxyconcanolide A [**21**]	ϵ_{243}: 23 200	*41*
9,23-di-*O*-acetyl-16-demethyl-21-deoxyconcanolide A [**22**]	ϵ_{242}: 28 500	*41*
21,23-dideoxy-23-*epi*-chloro-concanolide A [**23**]	ϵ_{244}: 35 100	*41*
9-*O*-[*p*-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-*epi*-[^{125}I]iodo−concanolide A [**24**]	ϵ_{243}: 56 144	this study

restricted the pharmacological use of these compounds, because the elimination of V-type ATPase activity also affected essential cellular processes such as iron, hormone, and nutrient uptake by receptor-mediated endocytosis (references given in refs *6* and *19*) and finally caused cell death (*21*−*24*).

Despite the intensive use of these macrolide antibiotics, their molecular mechanism of V-type ATPase inhibition is unknown. Investigations by Hanada et al. (*25*) implicated an interaction with the membrane-bound V_O part, which was confirmed in other studies with purified V_O complexes (*26*−*28*). However, the binding site remained undiscovered. The studies of Zhang et al. (*27*) suggested an interaction with the 116-kDa subunit of the V-type ATPase of clathrin-coated vesicles, while others suggested an interaction with the 17-kDa subunit (*26*, *29*). In addition, their inhibitory effect on P-type ATPases, e.g., the Kdp-ATPase of *Escherichia coli* (*7*, *8*, *30*), the Na^+,K^+-ATPase (*3*, *7*), the SR Ca^{2+}-ATPase (*7*), the H^+,K^+-ATPase (*31*), and the CadA of *Staphylococcus aureus* (*32*), has hardly been recognized. Studies with the Kdp-ATPase (*30*) suggest that bafilomycin A_1 inhibits the transition between the phosphorylated E_1P- and E_2P-forms that occurs during the reaction cycle of P-type ATPases (*33*). Therefore, these macrolides might also be useful tools for studying the reaction mechanism of P-type ATPase, at least for the Kdp-ATPase.

On the basis of our earlier studies with semisynthetic bafilomycin and concanamycin derivatives (*1*, *8*, *34*, *35*), we initiated detailed structure−activity investigations with semisynthetic concanamycin derivatives to approach the following two questions: (i) What is the pharmacophor, the structural key element, of the plecomacrolides that leads to their inhibitory potential against V- and P-type ATPases? (ii) Where is the binding site within these two different types of ATPases? In a first step, we examined where chemical modifications (O-acylations, substitutions, eliminations) could be placed without seriously affecting the inhibitory potential of the macrolides. In a second step, we used the knowledge of these structure−activity investigations to introduce traceable elements (fluorescent or radioactive) or nitrene-generating azido or carbene-generating diazirine-groups being able to bind the inhibitors to their target covalently (*36*). These studies led finally to the synthesis of two photoaffinity probes that were used in labeling experiments with the purified Kdp-ATPase of *E. coli* and the purified plasma membrane V-type ATPase of *Manduca sexta* (described in a following paper, ref *37*).

MATERIALS AND METHODS

Preparation of Vacuolar Membranes and Purification of the Kdp-ATPase. The Kdp-ATPase was purified as described previously (*38*) from the *E. coli* K12 strain TKA 1000 (*kdp*$^+$ Δ*atp706 nagA trkA405 trkD1 thi rha lacZ*). Vacuolar membranes of *Mesembryanthemum crystallinum* from plants grown in the CAM mode were prepared as described in Ratajczak (*39*); those of *Neurospora crassa* were obtained as detailed in Bowman and Bowman (*40*).

Concanamycin Derivatives. The purification of plecomacrolides and the synthesis of semisynthetic derivatives is described elsewhere (*36*, *41*, *42*). The structures of the plecomacrolides and their semisynthetic derivatives are shown in Figures 1−4. In general, the substances were dissolved at a concentration of 100 mM in DMSO. Aliquots of these solutions were diluted 1:10000 in methanol, and the actual concentration was determined photometrically using the molar extinction coefficients given in Table 1. This table is also a reference for the assignment of the numbers used in the text and figure legends to the chemical names of the compounds. From these primary solutions, several dilutions in DMSO were prepared; the dilutions contained a 50-fold excess of the desired inhibitor concentrations used in the ATPase activity tests (see below).

ATPase Assay. The ATPase activities of the purified KdpFABC complex of *E. coli* and of the V-ATPases of *M.*

crystallinum and *N. crassa* were analyzed with the automated ATPase microassay described by Henkel et al. (*43*). As a modification of the original procedure, the samples in the 96-well plates were measured in a SLT 340 ATTC micro ELISA plate reader using dual wavelength setting of 620 and 492 nm for test and reference filters, respectively. KH_2PO_4 solutions (0−7 nmol) were used as a double standard that occupied rows 1 and 2 of a microtiter plate. In contrast to the results reported by Henkel et al. (*43*), the ATPase microassay under the described conditions is linear in the range of 2.5 to 7 nmoles of ortho-phosphate. Before the assay was started by addition of different ATP solutions (see below), the microtiter plates were incubated for 5 min at 37 °C (Kdp-ATPase, V-ATPase of *M. crystallinum*) or 30 °C (V-ATPase of *N. crassa*) in a water bath or a specially manufactured aluminum heating block connected to a control unit (Unitek HB130), respectively. The plecomarolides were added from 50-fold stock solutions in DMSO to the cavities. In general, 1 μL was added to 24 μL of the enzyme-containing solutions. To these solutions (and the "ATP controls" that contained only the specific assay buffer), 25 μL of enzyme-specific ATP solutions (detailed below) were added with an 8-channel multipipet in time intervals of 10 s to rows 3−12 of the microtiter plate. Each row contained its own ATP control. In general, the ATPase reactions were stopped after exactly 5 min by the addition of 200 μL of the reaction mix (according to ref *43*) with another 8-channel multipipet. Because of this strategy, the samples in row 3 incubated 90 s longer in the presence of the very acidic reaction mix at 30 or 37 °C than those in row 12. When a time-dependent difference of the nonenzymatic ATP hydrolysis (ATP controls) was observed, the calculated activity was corrected by linear regression. If no time dependence was observed, the ATPase activity was corrected by the mean values of all ATP controls. For each inhibitor concentration tested, three different samples were analyzed. The determined absorption values were corrected by the "blank values", the fitted nonenzymatic ATP hydrolysis (ATP controls), and, if necessary, by the absorption of the enzyme or membrane-containing solutions (enzyme controls). The following buffer and ATP solutions were used (all values are final concentrations): (i) Purified Kdp-ATPase (0.55 μg/cavity): 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$; addition of 1 mM Na_2-ATP, pH 7.0; (ii) vacuolar membranes of *N. crassa* (1 μg protein/cavity): 10 mM PIPES-Tris, pH 7.5, 10 mM NH_4Cl/5 mM NaN_3/0.1 mM NH_4VO_3; addition of 2.5 mM Na_2ATP/2.5 mM $MgSO_4$; (iii) tonoplast membranes of *M. crystallinum* (0.5 μg of protein/cavity): 25 mM Tricine-Tris, pH 8.0/50 mM KCl/0.1 mM molybdate/0.002% Brij 58/0.1 mM $NaVO_4$/1 mM NaN_3; addition of 1.5 mM Na_2ATP/1.5 mM $MgSO_4$.

In case of the tonoplast membranes of *M. crystallinum*, the residual concanamycin-insensitive ATPase activity could be suppressed by the addition of 0.1 mM Na_3VO_4 and 1 mM NaN_3, which have no effect on the activity of the V-type ATPase (Ratajczak, personal communication). The addition of 1 μL of DMSO, the generally used solvent for the plecomacrolides, reduced the activity of the Kdp-ATPase by 5 to 10% and that of the V-type ATPases of *N. crassa* and *M. crystallinum* by 10−15% in comparison to DMSO-free controls. The activities of DMSO-containing samples, set as 100% activity, varied in different ATPase assays. Activities were the following: purified Kdp-ATPase 1.04−2.0 μmol mg^{-1} min^{-1} (mean 1.5 μmol mg^{-1} min^{-1}), V-ATPase of *N. crassa* 1.16−2.44 μmol mg^{-1} min^{-1} (mean 1.65 μmol mg^{-1} min^{-1}), and V-ATPase of *M. crystallinum* 1.62−4.37 μmol mg^{-1} min^{-1} (mean 2.52 μmol mg^{-1} min^{-1}).

Labeling Experiments with the Purified Kdp-ATPase. The specific radioactivity (10 mCi/mmol) of the semisynthetic 9-O-([3,5-^{3}H]-4-azidobenzoyl)-21-deoxy-concanamycin A [**17**] was determined by liquid scintillation counting using an external standard. The photoaffinity labeling of the Kdp-ATPase was carried out in Falcon 96-well microplates. Forty micrograms of the purified Kdp-ATPase were incubated for 5 min at 37 °C in the presence of 100 μM **17** in 50 mM Tris-HCl, pH 7.5, with or without 1 mM KCl, respectively. The samples were irradiated for 5 min with a Bachofer UV-lampe HL-15-M (2 × 15 W, wavelength 312 nm), which was placed 2.5 cm above the microplate. The Kdp subunits were separated on a 11% SDS polyacrylamide gel (*44*). The proteins were visualized with Coomassie Brillant Blue G250; the gel was stained and destained following the procedure of Weber and Osborn (*45*). Subsequently, the gel was incubated for 30 min in Amplyfi (Amersham), dried on filter paper, and subjected to direct autoradiography. The specific radioactivity (438.7 mCi/mmol) of the 9-O-[*p*-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-*epi*-[^{125}I]iodo-concanolide A [**24**] was determined in a γ-counter. The photoaffinity labeling of the Kdp-ATPase was carried out in Falcon 96-well microplates using 25 μg of the purified Kdp-ATPase incubated for 3 min at room temperature in the presence of 10 μM **24**. The samples were irradiated for 3 min with a Camag UV-lampe (17.6 W, wavelength 366 nm) as described above and separated using an SDS gradient gel (10−15% T, 3% C; ref *46*).

RESULTS

Standardized Test Conditions. On the basis of our finding that the concanamycins with their 18-membered macrolide-ring (Figure 1) are even better inhibitors of V-type ATPases than the 16-membered bafilomycins (*8*), we decided to use semisynthetic concanamycins for the development of photoaffinity derivatives of plecomacrolides. Our earlier work had shown that the plecomacrolides might interact stoichiometrically with vacuolar H^+-ATPases (*7*); therefore, the necessary concentration for half-maximal or complete inhibition depends on the amount and purity of V-type ATPase examined in the corresponding test (*7*, *25*, *47*−*49*). To screen a large variety of different substances under nearly identical conditions, we standardized the ATPase-activity test by using aliquots of the same preparation. For this purpose, it was necessary to minimize the quantity of the corresponding enzyme and membrane preparations per test. This was accomplished by the automated ATPase microassay described by Henkel et al. (*43*). A comparison of the automated ATPase microassay with the method used previously (*8*) gave almost identical results for the inhibition of the Kdp-ATPase by concanamycin A [**3**] (data not shown). In addition to the purified Kdp-ATPase of *E. coli* and the V-type ATPase (vacuolar membranes) of *N. crassa* that were used in our primary investigations (*7*, *8*), we included the V-type ATPase of *M. crystallinum* because this enzyme showed in an initial screening almost no difference between the naturally occurring plecomacrolides bafilomycin A_1 [**1**], bafilomycin B_1 [**2**],

Table 2: Inhibitory Effects of Plecomacrolide Derivatives on the ATPase Activity of the Purified Kdp-ATPase of *E. coli* and the V-Type ATPases of *N. crassa* and *M. crystallinum*

plecomacrolide[a]	Kdp-ATPase K_i [μM]/I_{50} [μmol mg^{-1}]	V-ATPase *N. crassa* K_i [nM]/I_{50} [μmol mg^{-1}]	V-ATPase *M. crystallinum* K_i [nM]/I_{50} [μmol mg^{-1}]
[**1**]	2.0/0.18	$5/0.5 \times 10^{-3}$	$1.6/0.32 \times 10^{-3}$
[**2**]	nt	$4/0.4 \times 10^{-3}$	$1.9/0.38 \times 10^{-3}$
[**3**]	1.2/0.11	$1.2/0.12 \times 10^{-3}$	$1.3/0.26 \times 10^{-3}$
[**4**]	ni[b]	$1.3/0.13 \times 10^{-3}$	$1.3/0.26 \times 10^{-3}$
[**5**]	6.2/0.56	5200/0.52	3000/0.6
[**6**]	ni	21100/2.11	11900/2.38
[**7**]	ni	8200/0.82	4200/0.84
[**8**]	ni	1800/0.18	1000/0.2
[**9**]	1.6/0.15	$1.2/0.12 \times 10^{-3}$	$0.6/0.12 \times 10^{-3}$
[**10**]	15/1.36	$0.9/0.09 \times 10^{-3}$	$1.2/0.24 \times 10^{-3}$
[**11**]	4.5/0.41	$8.5/0.85 \times 10^{-3}$	$1.2/0.24 \times 10^{-3}$
[**12**]	4.2/0.38	$0.9/0.09 \times 10^{-3}$	$1.1/0.22 \times 10^{-3}$
[**13**]	8.2/0.75	$6/0.6 \times 10^{-3}$	nt
[**14**]	7.2/0.65	$2.2/0.22 \times 10^{-3}$	nt
[**15**]	8.1/0.74	$11.8/1.18 \times 10^{-3}$	nt
[**16**]	3.2/0.29	$5.7/0.57 \times 10^{-3}$	$2.7/0.54 \times 10^{-3}$
[**17**]	7.9/0.72	$15/1.5 \times 10^{-3}$	$20/4 \times 10^{-3}$
[**18**]	5.4/0.49	$52/5.2 \times 10^{-3}$	195/0.039
[**19**]	7.5/0.68	$80/8.0 \times 10^{-3}$	2800/0.56
[**20**]	14.8/1.35	$18/1.8 \times 10^{-3}$	$40/8 \times 10^{-3}$
[**21**]	6.1/0.55	1600/0.16	400/0.08
[**22**]	1.2/0.11	300/0.03	200/0.04
[**23**]	1.9/0.17	$3.1/0.31 \times 10^{-3}$	$0.8/0.16 \times 10^{-3}$
[**24**]	40/4.1	nt	$22/0.22 \times 10^{-3}$ [c]

[a] The numbers assigned to the different plecomacrolides are given in Table 1. ni: no inhibition; nt: not tested. [b] At the maximal applied concentration of 100 μM, an inhibition of 20% was determined. [c] This set of data was obtained with vacuolar membranes of *Kalanchoë daigremontiana* that was inhibited by concanamycin A [**3**] with a K_i of 2.0 nM and an I_{50} of 0.02×10^{-3} μmol mg^{-1}.

concanamycin A [**3**], and concanamycin C [**4**] (Table 2). Furthermore, the higher affinity of the V-ATPase of *N. crassa* for concanamycins observed in our previous structure−activity investigations (*8*) was confirmed (Table 2). However, the 20-fold difference between bafilomycins and concanamycins that was observed previously (*8*) was reduced to a factor of 4 to 5. In independent studies with different *N. crassa* vacuolar membranes, a preparation-dependent fluctuation of this ratio was observed (E. J. Bowman, personal communication). However, the concanamycins were always better inhibitors than the bafilomycins. In contrast to these preparation-dependent fluctuations, V-type ATPases from other organisms/sources have shown a lesser (as in the case of *M. crystallinum*) or more pronounced (as in the case of *M. sexta*, see ref *37*) difference in inhibitor sensitivity toward concanamycins and bafilomycins (see Discussion).

Structure−Activity Investigations. The goal of the structure−activity investigations with further semisynthetic concanamycins was the synthesis of a traceable derivative that would be suitable for the specific covalent labeling of the V-type ATPases and the Kdp-ATPase. In a first step, we examined where chemical modifications (O-acylations, substitutions, eliminations) could be placed without seriously affecting the inhibitory potential of the concanamyins. In a second step, we used the knowledge of these structure−activity investigations to introduce traceable elements (fluorescent or radioactive) or nitrene-generating azido or carbene-generating diazirine groups. In this report, we present the results with 24 "key structures" (Figures 1−4) out of over 50 different derivatives tested (*36*).

Our previous studies (*8*) had shown that the 18-membered macrolide ring of the concanamycins (or the 16-membered ring of the bafilomycins, see Figure 1) plays a key role in the inhibition of V- and P-type ATPases, while the hemiketal

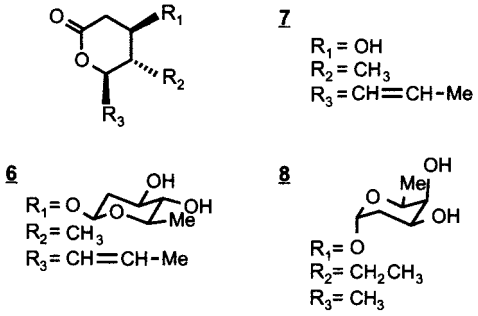
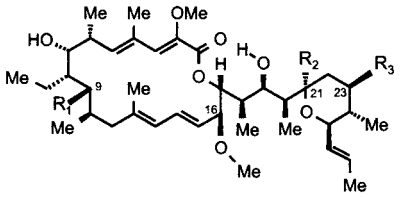


FIGURE 2: Structures of prelactones [**6**−**8**].

ring is necessary for maximal inhibition (Table 2). The additional sugar moiety of the concanamycins linked to the 23-hydoxyl group of the hemiketal ring (Figure 1) is not necessary for the biological activity but is responsible for the higher chemical stability of concanamycins (*8*). An involvement of the hemiketal ring in the interaction with V-type ATPases was confirmed by the inhibitory effect of the natural occurring "shunt product" prelactone C [**7**] (*34*) and the two semisynthetic prelactones **6** and **8** (*42*). These compounds (Figure 2), which correspond to an isolated hemiketal ring, inhibited both V-type ATPases at micromolar concentrations (Table 2, Figure 5), while an inhibitory effect on the Kdp-ATPase was not detected at a concentration of 200 μM (Table 2, Figure 5). In addition, elaiophylin [**5**], which showed no inhibition of the V-ATPase of *N. crassa* at concentrations up to 1 μM (*8*), inhibited both V-ATPases at higher concentrations (K_i values 3−5 μM; Table 2, Figure 5). The K_i value of **5** (6.2 μM) calculated from the inhibition of the Kdp-ATPase was almost identical to the K_i value reported previously (5.0 μM, ref *8*). By removal of the 21-OH group of **3**, **9** was obtained that showed almost identical effects as **3** on the three tested ATPases (Table 2). The



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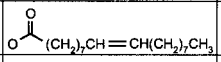
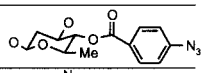
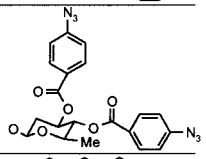
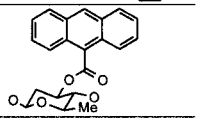
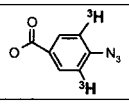
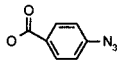
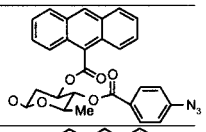
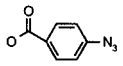
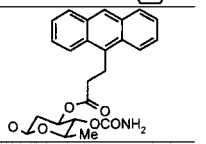
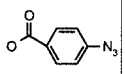
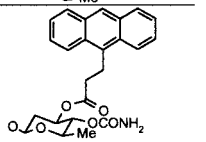
10	OH	H	OH
11	OH	H	
12		H	OH
13		H	
14	OH	OH	
15	OH	OH	
16	OH	OH	
17		H	
18		OH	
19		OH	
20	OAc		

FIGURE 3: Structures of semisynthetic concanamycins [**9**−**20**].

deglycosylation of **9** resulted in **10** (Figure 3). Because **10** retained the high inhibitory potential against V-type ATPases (Table 2), it was used for further modifications. Compound **10** was a suitable compound for modifications both because it is chemically more stable than concanamycins and because it contains a reduced number of chemically accessible hydroxyl groups (*41*). As a side product of the deglycosylation reaction of **9**, 16-demethyl-21-deoxyconcanolide A [**21**] was obtained. This derivative showed a drastically reduced affinity for both V-ATPases, while the inhibition of the Kdp-ATPase was moderately affected (Table 2). The diacetate [**22**] of **21** had similar effects (Table 2, Figure 5). In a next step, we modified 9-OH and 23-OH of **10**. The substitution of the 23-OH group by chlorine resulted in **23** (Figure 4), and the esterification of 9- or 23-OH with

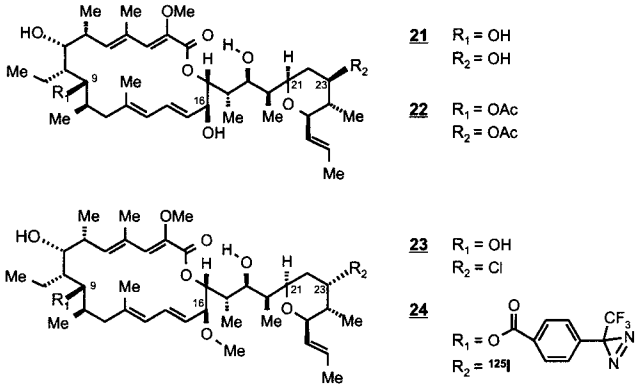


FIGURE 4: Structures of semisynthetic 21-deoxyconcanamycins [**21**−**24**].

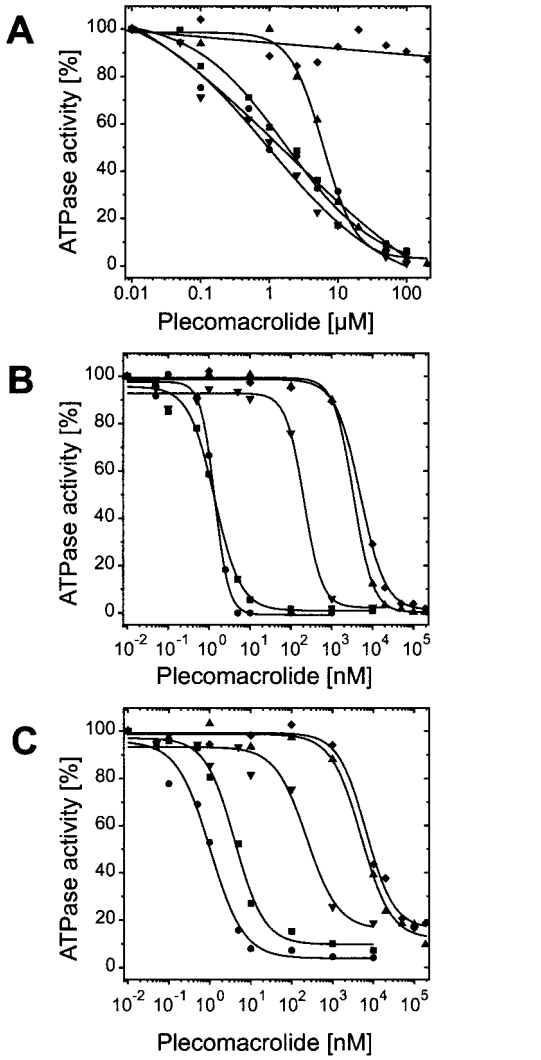


FIGURE 5: Inhibition of the Kdp-ATPase and two V-type ATPases by naturally occurring and semisynthetic plecomacrolides. Inhibitory effects of **1** (■), **3** (●), **5** (▲), **7** (◆), and **22** (▼) on (A) the purified Kdp-ATPase of *E. coli*, (B) the tonoplast V-type ATPase of *M. crystallinum*, and (C) the vacuolar V-type ATPase of *N. crassa*. Experimental procedures are detailed under Materials and Methods.

hydrophobic benzoic acids resulted in **11** and **12**, respectively (Figure 3). Neither one of these modifications nor the introduction of even larger hydrophobic aliphatic (as in **13**) or aromatic residues had a significant effect on the inhibition of the three different ATPases (Table 2 and data not shown).

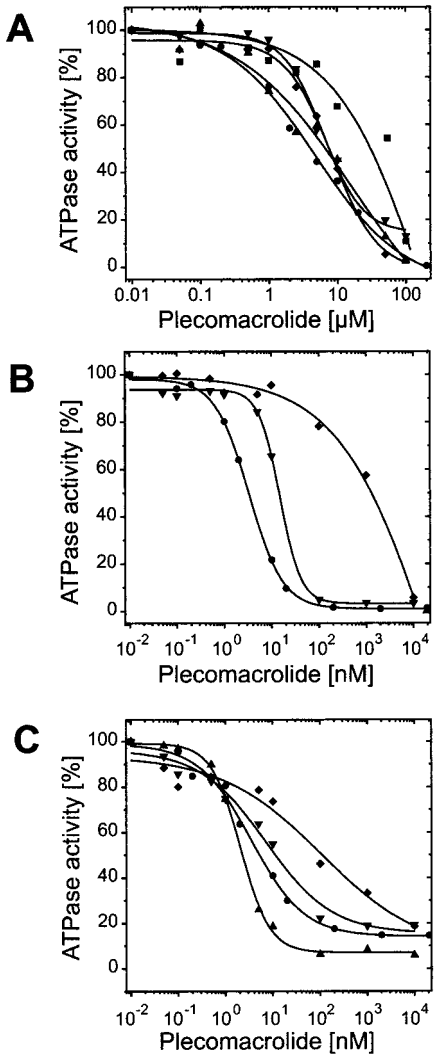


FIGURE 6: Inhibition of the Kdp-ATPase and two V-type ATPases by semisynthetic plecomacrolides that contain a traceable and/or a photoreactive group. Inhibitory effects of **14** (▲), **16** (●), **17** (▼), **19** (◆), and **24** (■) on (A) the purified Kdp-ATPase of *E. coli*, (B) the tonoplast V-type ATPase of *M. crystallinum*, and (C) the vacuolar V-type ATPase of *N. crassa*. Experimental procedures are detailed under Materials and Methods.

The next step was the introduction of traceable and "reactive" groups to establish a covalent linkage between the inhibitor and the enzyme. 4-Azidobenzoyl-groups (as in **14** and **15**) (Figure 3) or a 9-anthracenoyl-group (as in **16**) (Figure 3), bound to the sugar moiety of **4**, had no major effect on the inhibitory potential of the compounds (Table 2, Figure 6). With only traceable or only "reactive" derivatives, modification of the Kdp-ATPase or the V-type ATPases was not possible (results not shown). Therefore, we synthesized inhibitors [**17**−**20**, **24**] (Figures 3 and 4) that carried both a traceable and a "reactive" group. The fluorescent derivatives **18**−**20**, which contain an anthracene residue at the sugar moiety and an 4-azido-benzoyl group at 9-OH, showed drastically reduced effects on the Kdp-ATPase and especially on the two V-type ATPases (Table 2, Figure 6). Furthermore, no specific "photoaffinity-labeling" of the ATPases with one of the compounds was possible (data not shown). Since the introduction of a single bulky group at one of the chosen positions had only a moderate effect (see above), more than one bulky hydrophobic group was avoided in further derivatives. In **17**, both required functions were introduced by esterification with a tritium-labeled 4-azidobenzoic acid at position C-9 of **9**. Compound **17** showed only slightly reduced affinities for the Kdp-ATPase (K_i 7.9 μM) and the V-type ATPases (K_i 15 and 20 nM, respectively; Table 2, Figure 6). With this derivative, labeling of the Kdp-ATPase was obtained (see below), while labeling of V-ATPases was not observed, probably due to the relatively low specific radioactivity and the low traceability of tritium. Finally, we synthesized **24**, which contains a carbene-generating trifluoroethyl-diazirinyl-benzoyl-group at 9-OH of the macrolide ring and an ^{125}I at position C-23 of the hemiketal ring. This derivative inhibited the Kdp-ATPase with a K_i of 40 μM (Table 2, Figure 6) and was used for the photoaffinity-labeling of the Kdp-ATPase (see below) and the V-type ATPase of *M. sexta* (described in a following paper, ref *37*).

Photoaffinity-Labeling Experiments with the Purified Kdp-ATPase. In a first attempt, we used the nitrene-generating 9-*O*-([3,5-^{3}H]-4-azidobenzoyl)-21-deoxy-concanamycin A [**17**] for labeling experiments with the purified Kdp-ATPase and different V-type ATPases. This derivative binds to the KdpA and KdpB subunits (*50*). In contrast, covalent modification of only KdpB was observed when inverted vesicles from *E. coli* cells were used. In these experiments, covalent modification of an ATPase with a plecomacrolide derivative was shown for the first time. However, the relatively weak labeling was not reduced by the addition of an equal concentration of unmodified concanamycin A [**3**]. Therefore, the specificity of this covalent modification is questionable. Furthermore, the low specific radioactivity of **17** and the low energy of the β-irradiation of ^{3}H did not allow further analyses of the binding site within the Kdp-ATPase.

In a further attempt 9-*O*-[*p*-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-*epi*-[^{125}I]iodo-concanolide A [**24**] was used for labeling experiments with the purified Kdp-ATPase. The enzyme complex was incubated with and without KCl in the presence of 10 μM **24**. As already observed with **17**, this derivative reacted with the subunits KdpA and KdpB. However, neither concanamycin A [**3**] nor bafilomycin B_1 [**2**] quenched this covalent modification (data not shown). Thus, the specificity of this covalent modification is ambiguous.

DISCUSSION

The discovery of the bafilomycins and concanamycins as high-affinity inhibitors of V-ATPases (*7*, *8*) paved the way for detailed investigations of the physiological role of these proton pumps. These pumps are widespread in endosomes and lysosomes of eukaryotic cells, in vacuoles of plants and fungi, and in the plasma membranes of specific animal cells (overview in ref *51*). Despite the common use of the plecomacrolides as V-ATPase inhibitors, their binding site and the molecular mechanism of action remained unknown. Primary studies suggested a noncovalent binding (*28*) to the membrane-embedded V_O-part of these multisubunit proton pumps (*25*−*27*). In addition, bafilomycins and concanamycins inhibit some P-type ATPase (see above) in micromolar concentrations but do not affect the ATP synthases (or F-ATPases), which are structurally homologous to the V-ATPase (*7*, *8*). These findings raised the question of how

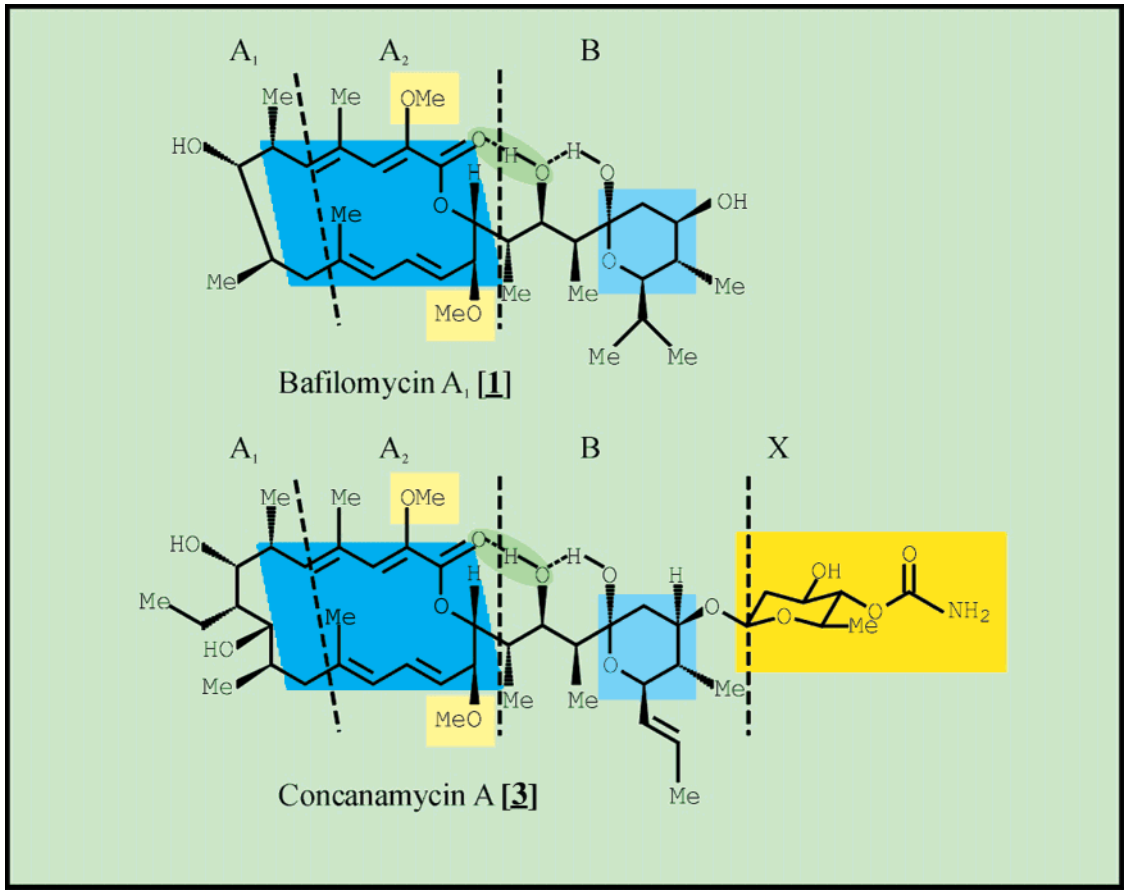


FIGURE 7: Structural elements of bafilomycins and concanamycins that affect the inhibitory potential against V-type ATPases. Shown are the structures of bafilomycin A_1 [**1**] and concanamycin A [**3**]. The demarcation of the structural segments A_1, A_2, B, and X is according to Bindseil (*60*). On the basis of substantial structure−activity investigations with different V-type ATPase, the most important part of the plecomacrolides is the invariant segment A_2 of the closed macrolactone ring (cyan). The methylation of the two hydroxyl groups of A_2 (light yellow), as well as the hydrogen bond (green) between the CO- and a hydroxyl group of the spacer, are important to keep the segment A_2 in the most effective conformation. The hemiketal ring (blue) is necessary for optimal inhibition and may interact with a secondary binding site. The sugar residue (yellow) of some plecomacrolides is not involved in direct inhibitor/enzyme interactions.

the bafilomycins and concanamycins exert their effect at the molecular level and whether similar binding sites exist within the V- and P-type ATPases. To determine the binding site(s) within these two groups of ATPases, we set out to synthesize a derivative of these antibiotics that would be capable of forming a covalent bond to the ATPases. As a prerequisite, we determined which sites within the plecomacrolides could be modified without losing their inhibitory potential.

In several studies, including our primary investigations (*8*), structure−activity investigations were carried out with different chemically modified (semisynthetic) or naturally occurring bafilomycins and concanamycins to identify "important elements" that determine the inhibitory potential against V-type ATPases. The similar potency of these macrolide antibiotics is based on their high overall structural similarity (Figure 7) and the identity of comparable chiral centers (*1*, *34*). These facts allow a collective discussion of structure−activity investigations of concanamycin and bafilomycin derivatives. The slightly higher affinity of concanamycins as compared with bafilomycins to V-type ATPases determined in our previous investigations (*8*) was confirmed in other comparative studies with V-ATPase of fungi and animal cells (*52*−*55*). However, the 20-fold higher potency observed previously (*8*) was never reached again. In general, concanamycin A [***3***] inhibited V-ATPases 2−5-fold more effectively than bafilomycin A_1 [**1**] (see also Table 2, inhibition of the V-ATPase of *N. crassa*). This higher potency was linked to the higher conformational flexibility of the 18-membered macrolide ring of the concanamycins, which led to the proposal of an "induced fit" model (*1*). In contrast, in studies with the V-ATPase of *M. crystallinum*, virtually no significant differences were observed in the inhibitory effect of the four naturally occurring bafilomycins A_1 [**1**] and B_1 [**2**], and the concanamycins A [**3**] and C [**4**]. It is worth mentioning that these tests were performed with the same inhibitor solutions as those with the V-ATPase of *N. crassa*, where differences were clearly detected. Whether the *M. crystallinum* pump is the "exception to the rule" or whether this represents a characteristic of plant V-ATPases has to await further investigations. However, in general, specific chemical modifications of the concanamycins had a comparable effect on V-type ATPases of *N. crassa* and *M. crystallinum* (Table 2).

The basic conclusions drawn from our primary structure−activity studies and from those of other groups could be confirmed and further extended by this report: The macrolactone ring of the plecomacrolides contributes strongly to the inhibition potential, since derivatives with an open hemiketal ring still inhibit V- and P-ATPases (*8*, *18*, *21*, *56*, *57*); however, the lower affinity of these derivatives indicates that a closed hemiketal ring is necessary for optimal

inhibition. The participation of the hemiketal ring in the enzyme−inhibitor interaction can also be deduced from the fact that the shunt product prelactone C [**7**] and the two related compounds **6** and **8**, which represent "isolated hemiketal rings", inhibit V-ATPases at micromolar concentrations. In addition, Gagliardi et al. (*18*) reported that in an in vitro test a bafilomycin derivative with an open macrolactone ring, but carrying an intact hemiketal ring (bafilomycin V_1), inhibited two different V-ATPases in submicromolar concentrations. In contrast, this (*58*) or related plecomarolides with an open macrolactone ring (*21*, *57*) had no inhibitory effect in cell cultures. The additional sugar moiety in concanamycins, which is absent in most bafilomycins, does not play an essential role for the inhibitory action (*8*, *21*, *56*, *57*, and this report).

There are indications that the conformation of the macrolactone ring has an important function for the inhibitory action against V-type ATPases. The cleavage of the 16-methoxy-group resulted in a drastically reduced potency of the corresponding 16-demethyl-derivatives **21** and **22**. Probably, the free 16-OH disturbs the hydrogen bonding system between 19-OH and 1-CO resulting in the twisting of the structural element B (see Figure 7), which has most likely an effect on the overall conformation of the macrolactone ring (*41*). Therefore, the methylation of the 16-OH group, and probably also that of the 2-OH group on the other side of the ring system, does not allow the development of an alternative hydrogen bonding system between the macrolactone ring and the adjacent side chain that would finally result in a less active conformation of the plecomacrolides (comparable methoxy groups also exist in the bafilomycins). Hence, the hydrogen bond between the 19-OH and the 1-CO and the methylation of adjacent hydroxyl groups are very important to keep the macrolactone ring in the most active conformation. Interestingly, these methyl groups are added in an independent step after the synthesis of the plecomacrolides by a type I polyketide synthase (*1*). Also other modifications, like the total hydrogenation of the dienic system of the macrolactone ring, that most likely have an effect on the conformation, decrease the inhibitory potential against V-ATPases drastically (*18*). In contrast to the hydrogen bond mentioned above, the one between the 21-OH of the hemiketal ring and the 19-OH of the spacer has no influence on the inhibitory potential of the plecomacrolides, since methylation or acylation of the 21-OH (*8*) or its removal, as in **9**, had little or no effect. Similar results were obtained with corresponding bafilomycin-derivatives (*8*, *18*). Therefore, a specific conformation of the hemiketal ring, which probably exists in the natural plecomacrolides due to that hydrogen bond, is not essential for maximal activity. In addition, several chemical modifications at the hemiketal ring could be performed without any significant alterations of the inhibitory potential, as long as the six-membered ring stays intact. Again, similar conclusions were drawn from structure−activity investigations with comparable bafilomycin derivatives (*18*).

Chemical modifications of the 9-OH group, the most reactive group of the macrolactone ring, were also well tolerated. At this position, large hydrophobic groups could be introduced without severely affecting the inhibitory potential against both groups of ATPase. However, the combined introduction of two bulky hydrophobic groups at 9-OH and at hydroxyl groups of the hemiketal ring or the attached sugar, as in **18**−**20**, drastically reduced the inhibitory potential. Most likely, two hydrophobic groups altered the conformation and/or solubility of the corresponding derivatives such that the interaction with the enzyme complexes was impaired. Except **19** (in the case of *M. crystallinum*), all other photoaffinity probes have K_i values that are at least an order of magnitude lower than those of elaiophylin [**5**] and the prelactones [**6**−**8**] (see Table 2). Therefore, we conclude that they were still capable of exerting (weak) interactions with the primary binding site (see below).

Further conclusions can be drawn from the inhibitory effect of elaiophylin [**5**], which inhibited the V-ATPases of *M. crystallinum* and *N. crassa* in micromolar concentrations. In our previous studies, no inhibitory effect on the *N. crassa* V-type ATPase was discovered, because only concentrations up to 1 μM were tested (*8*). Independent studies confirmed that elaiophylin [**5**] has no inhibitory effect upon V-type ATPases at a concentration of 50 nM (*55*). However, the inhibition occurred at a concentration where its cleavage product **8**, which represents one hemiketal ring, is also active. Therefore, the hemiketal ring, which is similar to those of bafilomycins and concanamycins, is most likely the part of elaiophylin [**5**] that determines the inhibitory potential against V-ATPases. This is not surprising since the symmetrically organized elaiophylin possesses a drastically changed molecular geometry with a 16-membered macrodiolide ring, two slightly modified hemiketal rings, two hydrogen bond systems, and two hydrophilic sugar residues on both sides of the macrolactone ring (Figure 1). These hydrophilic parts at both sides of the molecule probably prevent the insertion of this plecomacrolide into a hydrophobic environment. Therefore, the most active part of the plecomacrolides, the "A_2-B part" of the macrolactone ring (see Figure 7), may not reach its binding site (see below). Taking the investigations with different plecomacrolides together, we propose that a primary and a secondary binding site exist in V-ATPases and that these two sites interact with different parts of one plecomacrolide molecule. One site possesses a hydrophobic environment, probably the 17-kDa subunits of V_O (see a following paper, ref *37*), which interacts with the macrolactone ring of the plecomacrolides. This site determines the major enzyme−inhibitor interaction, and binding to this site is sufficient for inhibition, since derivatives without an opened hemiketal ring can still inhibit V-type ATPases completely. A secondary site contacts the hemiketal ring of plecomacrolides such as bafilomycin A_1 [**1**] or concanamycin A [**3**]. Individually, this additional interaction is relatively weak, as judged from the inhibition of V-type ATPase by prelactones, but it enhances the inhibitory potential of the bound plecomacrolide molecule. Also binding to this secondary site is sufficient for complete inhibition, but because of the much lower affinity, much higher concentrations (3−4 orders of magnitude) of prelactones are necessary. Because elaiophylin is able to reach this secondary site, it probably lies within a more hydrophilic environment. Candidates for this secondary site are subunits of the two stalk elements that connect the membrane-bound V_O with the catalytic subunits of V_1 (*59*).

The inhibitory mechanism of plecomacrolides on the Kdp-ATPase is at least in part different from that described above. The structure−activity investigations have shown that an

intact macrolactone ring per se is of extreme importance, while an intact hemiketal ring is indispensable for maximal inhibition. However, an inhibitory effect of the prelactones was not detected, while elaiophylin was a relative good inhibitor of the Kdp-ATPase. In addition, a demethylation of the 16-methoxy group, which alters the conformation of the macrolactone ring, had no significant effect. On the other hand, the addition of hydrophobic groups decreased the inhibitory potential. Taken together, the inhibition of the Kdp-ATPase depends on similar structural elements of the plecomacrolides that are necessary for the inhibition of the V-type ATPase, but the inhibitory potential is more affected by the general hydrophobicity than by a distinct conformation. Because the effect of the elaiophylins is comparable to that of the bafilomycins and the concanamycins, we postulate that the binding site(s) within the Kdp-ATPase is more hydrophilic than that of the V-type ATPases. The identification of the plecomacrolide binding site within Kdp has thus far failed. The K_i values of the plecomacrolides are in general relatively high (in the micromolar range), thereby increasing the possibility of unspecific labeling. In addition, the specific radioactivity of **17** and **24** was relatively low. To detect labeling at all, a high inhibitor concentration was necessary, which decreases the scope of quenching experiments with unlabeled plecomacrolides. However, the experiments with the Kdp-ATPase showed for the first time, that covalent labeling with our designed photoaffinity probes was possible. Therefore, these experiments were an important step for the successful photoaffinity labeling of the purified V-type ATPase of *M. sexta* (see a following paper, ref *37*).

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