Fluorescent Derivatives of Strophanthidin. Interaction with Sodium- and Potassium-Activated Adenosine Triphosphatase

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Four 3-*O*-acyl strophanthidins and 11 19-hydrazone derivatives were synthesized and tested as inhibitors of Na^+- and K^+-activated ATPase. The 3-acyl derivatives were much more active inhibitors than the 19-hydrazones. The 3-salicylate and 19-salicylhydrazone derivatives of strophanthidin possess strong fluorescence and might serve as fluorescent labels for studies on the purification of Na^+- and K^+-ATPase. The 3-salicylate exhibits altered fluorescent properties in the presence of a Na^+- and K^+-activated ATPase preparation.

Cardioactive steroids, such as strophanthidin and their glycosides, are of therapeutic importance for the treatment of heart diseases. Investigations on the molecular basis for their cardiotonic activity are as yet incomplete, but, at least in part, their pharmacological effects appear related to inhibition of the membranal Na^+- and K^+-activated ATPase system.[1,2] This inhibition which has been extensively studied results from a nearly irreversible binding of the cardioactive steroid to one conformation of the enzyme.[3]

Solubilization and purification of Na^+- and K^+-ATPase has proven difficult. The enzyme has been labeled with radioactive phosphate[4] or with radioactive cardiac steroids[5] and subsequent fractionation has been measured radiometrically. Early studies in this direction explored the alkylation of the enzyme with 3-haloacetates of such steroids.[6] Recently the 15-bromoacetate was reported to alkylate the enzyme,[7] while related 21-haloacetates were inactive.[8] An alternate method of labeling the active site of Na^+- and K^+-ATPase could involve a fluorescent derivative of strophanthidin. Such a derivative might also serve as a reporter or probe to study the nature of the active site.[9] Preliminary studies with 1-anilino-8-naphthalenesulfonic acid and Na^+- and K^+-ATPase[10] revealed the utility of such an approach.

The present report describes the attempt to devise a fluorescent derivative of strophanthidin for use as a fluorescent monitor for the purification of Na^+- and K^+-ATPase. Ideally such a substrate should exhibit an altered fluorescent spectrum on binding to the enzyme. Since the 3-hydroxy group and the 19-aldehyde group do not appear essential to the activity of strophanthidin,[1] these preliminary investigations have dealt with the synthesis of various 3-esters and 19-hydrazones and their activity as inhibitors of Na^+- and K^+-ATPase. During the course of this research an abstract appeared which reports on the enhancement of the fluorescence intensity of the 19-sulfonaphthylhydrazone of hellebrigenin on binding to Na^+- and K^+-ATPase.[11]

Experimental Section†

The Na^+- and K^+-ATPase preparation from *Electrophorus* electric organ[12] was a gift from Dr. R. W. Albers. Magnesium adenosine 5′-triphosphate, type I lactic dehydrogenase, and strophanthidin (Grade I) were from Sigma Chemical Co. The 2-phosphoenolpyruvate tricyclohexylammonium salt was from Calbiochem.

Thin-layer chromatography on silica gel GF was routinely used for purification (plates 20 cm × 20 cm and 1 mm thick) and for ascertaining the purity of the final products. Detection was with short-wave uv light and the developing solvent was $CHCl_3$–MeOH–EtOAc (6:3:1) unless otherwise indicated. Elution was with $CHCl_3$–MeOH (1:1). Melting points were taken on a Kofler hot stage and are corrected. Several compounds, though homogeneous on tlc, did not exhibit sharp melting points. Optical rotations were obtained on a Perkin-Elmer polarimeter (Model 141).

Assay of Na^+- and K^+-ATPase. Spectrophotometric assay[3] of Na^+- and K^+-ATPase was conducted in a solution consisting of 0.27 m*M* disodium DPNH, 0.72 m*M* tricyclohexylammonium phosphoenolpyruvate, 3 m*M* $MgCl_2$, 25 m*M* KCl, 40 m*M* Tris–HCl buffer (pH 7.3), 5 m*M* dithiothreitol, and 0.7 m*M* magnesium ATP and containing 7.5 units of pyruvate kinase and 7.5 units of lactic dehydrogenase in a final volume of 0.83 ml. Unspecific hydrolysis of ATP was initiated by addition of 0.03 unit of Na^+- and K^+-ATPase in a preparation containing some unspecific ATPase.[12] The rate of ADP formation was measured by recording the rate of NADH disappearance spectrophotometrically at room temperature for 2 min. The specific activity of Na^+- and K^+-ATPase was calculated from the difference between this rate (0.05 μmole/min per mg of protein) and the 50-fold higher rate after adding 10 μl of 5 *M* NaCl on a vigorously agitated microspoon. The reaction became linear 40 sec after the addition of NaCl and was then 2.47 μmoles/min per mg of protein. Deviation from perfect linearity was <10% during the following 4 min. If 5–10 μl of ethanol was added, the deviation from linearity was not significantly higher (<15%). The inhibitor was added in 5–10 μl of ethanol on a microspoon 1.5 min after the addition of NaCl. The rate of hydrolysis was then measured between 2.5 and 3 min after addition of the inhibitor. The specific inhibition of Na^+- and K^+-ATPase was calculated by comparing this rate to the rate of a control in which only ethanol was added.

Strophanthidin 3-Benzoate. When 0.4 g (2.86 mmoles) of benzoyl chloride was added to a soln of 100 mg (0.25 mmole) of strophanthidin in 2.0 ml of pyridine, there was an immediate pptn of crystals. The mixt was allowed to stand at room temp for 18 hr, after which it was poured into ice water. The mixt was then extd twice with $CHCl_3$. After washing with 5% Na_2CO_3 and water, the $CHCl_3$ ext was evapd under reduced pressure. Crystallization of the residue from dilute MeOH gave 68 mg (53%) of tan crystals, mp 216–220°. After two additional recrystallizations, the mp was 227–231°; $[\alpha]^{20}D$ +50.6° ($CHCl_3$); lit.[13] mp 230°; $[\alpha]^{26}D$ +47.8° ($CHCl_3$).

Strophanthidin 3-(*p*-Methoxybenzoate). To a soln of 96 mg (0.24 mmole) of strophanthidin in 1.0 ml of pyridine was added 0.2 ml of *p*-methoxybenzoyl chloride. The mixt was allowed to stand at room temp for 18 hr and then extd as described for the 3-benzoate. The residue was recrystallized from MeOH to give 86 mg (69%) of a colorless solid. After two recrystallizations from EtOH, the compound melted between 180 and 225° dec; $[\alpha]^{20}D$ +34.8° ($CHCl_3$). *Anal.* ($C_{31}H_{38}O_8$) C, H.

Strophanthidin 3-Cinnamate. To a cold soln of 100 mg (0.25 mmole) of strophanthidin in 2.0 ml of pyridine was added 200 mg (1.2 mmoles) of cinnamoyl chloride. The mixt was allowed to stand 18 hr at room temp and then extd as described for the 3-benzoate. The residue was crystallized twice from dilute MeOH to give 18 mg of pale yellow crystals, melting range 208–220° (two-phase melt); $[\alpha]^{20}D$ +48.0° ($CHCl_3$). *Anal.* ($C_{32}H_{38}O_7$) C, H.

Strophanthidin 3-Salicylate. The conversion of salicyclic acid into salicyl chloride, though unsuccessful with oxalyl or thionyl chloride, gave the desired acid chloride when thionyl chloride was used in the presence of a catalytic amount of aluminum chloride.[14] A mixt of 1.0 g (7.2 mmoles) of salicylic acid, 0.7 ml of thionyl chloride, and 20 mg of aluminum chloride was placed in a bath (45–50°) for 1.5 hr. Gas was evolved throughout this period. Excess thionyl chloride was then removed *in vacuo*. The product was mixed two times with dry benzene and the solvent removed *in vacuo*. The semisolid salicyl chloride was then dried at room temp under high vacuum.

†Where analyses are indicated only by symbols of the elements, analytical results obtained were within ±0.4% of the theoretical values.

A soln of 200 mg (0.5 mmole) of strophanthidin in 1.5 ml of pyridine was added to 0.5 g (3.2 mmoles) of salicyl chloride in 1.5 ml of pyridine. The resulting soln was then placed in an oil bath (90–100°) for 18 hr. After cooling, the reaction mixture was poured into ice water and then extd three times with $CHCl_3$. The ext was washed with water and dried (Na_2SO_4). Tlc analysis of the reaction mixture indicated *ca.* 20% formation of the desired ester. The mixt was purified on preparative tlc plates with $CHCl_3$–MeOH–EtOAc (7:2:1). The uppermost uv-absorbing band was eluted to give 41.6 mg of a solid, homogeneous by tlc. Recrystallization of the product from EtOH gave 14 mg of light tan rosettes; mp 232–234°; $[\alpha]^{20}D$ +56.2° ($CHCl_3$). The mass spectrum exhibited a parent ion at *m/e* 524 ($C_{30}H_{36}O_8$); infrared ($CHCl_3$) 1675 cm^{-1} (*o*-hydroxybenzoate ester).

Strophanthidin 19-Phenylhydrazone. A soln of 100 mg (0.25 mmole) of strophanthidin and 50 mg (0.46 mmole) of phenylhydrazine in 1.5 ml of absolute EtOH was allowed to stand at room temp for 18 hr. The soln was then transferred to an evaporating dish and after 6 hr the large crystals were recrystallized from dilute EtOH to give 103 mg (83%) of bright yellow flakes, mp 245–248°. After two recrystallizations the mp was 248–250°; $[\alpha]^{20}D$ −6.6° ($CHCl_3$); lit.[15] mp 230–232°; $[\alpha]^{20}D$ −5° (compound contains $2H_2O$ of crystallization).

Strophanthidin 19-[(4-Hydroxy-6-methyl)-2-pyrimidylhydrazone]. A mixt of 100 mg (0.25 mmole) of strophanthidin and 55 mg (0.39 mmole) of 2-hydrazino-4-hydroxy-6-methylpyrimidine in 3.0 ml of EtOH and 0.5 ml of AcOH was refluxed for 2 hr and then allowed to stand at room temp overnight. After evaporation, the residue was recrystallized from EtOH to give 56 mg (42%) of colorless needles; mp 225–235°; $[\alpha]^{20}D$ +166° (C_2H_5OH). *Anal.* ($C_{28}H_{38}N_4O_6$) N.

Strophanthidin 19-Indole-3-acetylhydrazone. A soln of 100 mg (0.25 mmole) of strophanthidin and 57 mg (0.3 mmole) of indole-3-acetylhydrazide in 2.0 ml of EtOH containing 10% AcOH was allowed to stand at room temp for 46 hr. The soln was then evaporated under reduced pressure, mixed with EtOH, and evaporated. After preparative tlc, the major uv-absorbing zone was eluted to yield 111 mg of a solid, homogeneous by tlc. Crystallization from acetonitrile gave 50 mg (34%) of a pale yellow solid; mp 190–218°; $[\alpha]^{20}D$ +79.2° (C_2H_5OH). *Anal.* ($C_{33}H_{41}N_3O_6$) N.

Strophanthidin 19-Salicylhydrazone. A soln of 100 mg (0.25 mmole) of strophanthidin and 45 mg (0.3 mmole) of salicylhydrazide in 2.0 ml of EtOH containing 10% AcOH was allowed to stand at room temp for 3 days. After evapn the residue was crystallized from acetonitrile to afford 58 mg (43%) of a tan solid, softening at 195° with melting complete at 210°; $[\alpha]^{20}D$ +65.7° (C_2H_5OH). *Anal.* ($C_{30}H_{38}N_2O_7$) N.

Strophanthidin 19-(*p*-Hydroxybenzoylhydrazone). When a mixt of 100 mg (0.25 mmole) of strophanthidin and 46 mg (0.3 mmole) of *p*-hydroxybenzoylhydrazide in 4.0 ml of EtOH containing 10% AcOH was refluxed for 5 min, a clear soln was obtained which was allowed to stand at room temp for 90 hr. The residue after evapn consisted of one major and two minor products. After preparative tlc, the major uv-absorbing zone was eluted to yield 110 mg of a solid. Tlc showed the compound was still contaminated. After another preparative tlc, elution of the lower portion of the uv-absorbing zone gave 88 mg of pure product, homogeneous by tlc. Crystallization from MeCN–MeOH gave 55 mg (41%) of a pale yellow solid, which slowly melted from 236°; $[\alpha]^{20}D$ +53.5° (C_2H_5OH). *Anal.* ($C_{30}H_{38}N_2O_7$) N.

Strophanthidin 19-(2-Pyridylhydrazone). A soln of 100 mg (0.25 mmole) of strophanthidin and 33 mg (0.30 mmole) of 2-hydrazinopyridine in 212 ml of EtOH containing 10% AcOH was allowed to stand at room temp for 90 hr. The residue after evapn was subjected to preparative tlc. Elution of the lower two-thirds of the major uv-absorbing zone gave 54 mg of pure product. Crystallization from MeCN gave 29 mg (23%) of yellow rod-shaped crystals; mp 199–201°; $[\alpha]^{20}D$ +107° (C_2H_5OH). *Anal.* ($C_{28}H_{37}N_3O_5$) N.

Strophanthidin 19-(2-Benzothiazolylhydrazone). A soln of 125 mg (0.32 mmole) of strophanthidin and 63 mg (0.38 mmole) of 2-hydrazinobenzothiazole in 4.0 ml of EtOH containing 10% AcOH was allowed to stand at room temp for 70 hr. The residue after evapn was recrystallized twice from EtOAc–MeOH to give 85 mg (48%) of light tan needles; mp 241–242°; $[\alpha]^{20}D$ +24.1° ($CHCl_3$). *Anal.* ($C_{30}H_{37}N_3O_5S$) N.

Strophanthidin 19-(2-Quinolylhydrazone). A soln of 125 mg (0.32 mmole) of strophanthidin and 60 mg (0.38 mmole) of 2-hydrazinoquinoline in 2.5 ml of EtOH containing 10% AcOH was allowed to stand at room temp for 70 hr. The residue after evapn was subjected to preparative tlc. Elution of the lower three-fourths of the major uv-absorbing zone gave 183 mg of a solid. Crystallization from EtOAc–MeOH gave 69 mg (40%) of greenish yellow spheres; mp 192–194°, a viscous melt with loss of viscosity at 205°; $[\alpha]^{20}D$ +69.5° (C_2H_5OH). *Anal.* ($C_{32}H_{39}N_3O_5$) N.

Strophanthidin 19-(3-Quinolylhydrazone). A mixt of 125 mg (0.32 mmole) of strophanthidin and 88 mg (0.38 mmole) of 3-hydrazinoquinoline dihydrochloride in 20 ml of EtOH and 0.5 ml of AcOH was placed in an oil bath (70°) for 6 days. EtOH was added periodically to maintain the original volume. The residue after evapn was applied to alumina GF preparative tlc plates and developed with $CHCl_3$–MeOH–EtOAc (8:1:1). Elution of the lower uv-absorbing zone gave 86 mg of a pale yellow solid. Crystallization from C_6H_6–EtOAc gave 58 mg (33%) of tan-colored solid; mp 193–203°; $[\alpha]^{20}D$ +132° (C_2H_5OH). *Anal.* ($C_{32}H_{39}N_3O_5$) N.

Strophanthidin 19-(6-Purinylhydrazone). A soln of 125 mg (0.32 mmole) of strophanthidin and 57 mg (0.38 mmole) of 6-hydrazinopurine in 5.5 ml of EtOH–AcOH (1:1) was allowed to stand at room temp for 3 days. After evapn the residue was subjected to preparative tlc. Elution of the upper uv-absorbing zone afforded 180 mg which on crystallization from MeCN–MeOH gave 76 mg (42%) of colorless crystals. After recrystallization, the compound melted slowly from 244°; $[\alpha]^{20}D$ +67.8° (C_2H_5OH). *Anal.* ($C_{28}H_{36}N_6O_5 \cdot CH_3OH$) C, H, N.

Strophanthidin 19-(1-Phthalazylhydrazone). A soln of 125 mg (0.32 mmole) of strophanthidin and 61 mg (0.38 mmole) of 1-hydrazinophthalazine in 2.7 ml of EtOH and 0.7 ml of AcOH was allowed to stand at room temp for 3 days. After evapn, the residue was applied to alumina GF preparative tlc plates, and developed in

Table I. Inhibitory Effect of Strophanthidin Derivatives on Na^+- and K^+-Activated ATPase. For Assay see Experimental Section

Inhibitor	% control at inhibitor concentration (*M*)			Fluorescence
	6×10^{-5}	3×10^{-6}	1.2×10^{-6}	
Strophanthidin	0	7	38	
Strophanthidin 3-benzoate	0.3	11	52	
Strophanthidin 3-(*p*-methoxybenzoate)	1	27	68	
Strophanthidin 3-cinnamate	1	12	56	
Strophanthidin 3-salicylate	0.3	9	48	Strong
Strophanthidin 19-phenylhydrazone	75			
Strophanthidin 19-[(4-hydroxy-6-methyl)-2-pyrimidylhydrazone]	87			
Strophanthidin 19-(indole-3-acetylhydrazone)	72			
Strophanthidin 19-salicylhydrazone	27			Strong
Strophanthidin 19-(*p*-hydroxybenzoylhydrazone)	80			
Strophanthidin 19-(2-pyridinylhydrazone)	83			
Strophanthidin 19-(2-benzothiazolylhydrazone)	52			
Strophanthidin 19-(2-quinolylhydrazone)	21			Weak
Strophanthidin 19-(3-quinolylhydrazone)	36			Weak
Strophanthidin 19-(6-purinylhydrazone)	85			
Strophanthidin 19-(1-phthalazylhydrazone)	89			

Table II. Fluorescence Properties of Strophanthidin 3-Salicylate[a]

Solvent[b]	Enzyme prepn,[c] μl/ml	Emission, nm	Relative quantum yield[d]
Water (0.2% ethanol)		385	5.5
Hexane (0.1% ethanol)		340	7.3
Water (0.2% ethanol)	1	380	5.8
Water (0.2% ethanol)	3	370	6.1
Water (0.2% ethanol)	5	362	6.8
Water (0.2% ethanol)	10	348	7.2

[a] A solution of 10 μg/ml was excited at 306 nm and the emission spectrum was recorded with an Aminco Bowman spectrofluorimeter. [b] Deionized water, glass-distilled three times, spectro quality hexane (Matheson Coleman and Bell). The solvent mixtures showed no emission between 330 and 550 nm when excited at 306 nm. [c] A Na^+- and K^+-ATPase preparation from *Electrophorus* electric organ[12] containing 12 μg of protein/μl was used. At this concentration of strophanthidin 3-salicylate, light scattering prevented the use of more than 10 μl/ml of enzyme. [d] Expressed as parts per thousand of the emission of an equivalent concentration of quinine·2HCl in 1 *N* H_2SO_4 (exciting wavelength 348 nm, emitting wavelength 450 nm).

Table III. Fluorescence Properties of Strophanthidin 19-Salicylhydrazone[a]

Solvent[b]	Enzyme prepn,[c] μl/ml	Emission, nm	Relative quantum yield[d]
Water (0.5% methanol)		430	10.0
Benzene (0.5% methanol)		464	4.0
Water (0.5% methanol)	3	430	9.4
Water (0.5% methanol)	10	430	7.6
Water (0.5% methanol)	30	430	7.0

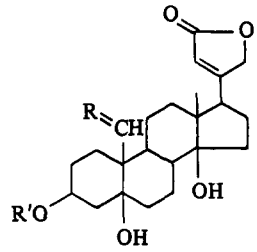
[a] A solution of 50 μg/ml was excited at 340 nm and the emission spectrum was recorded with an Aminco Bowman spectrofluorimeter. [b] Deionized water glass-distilled three times, spectro quality methanol (Matheson Coleman and Bell), and reagent grade benzene (Baker Chemical Co.) were used. The solvent mixtures used showed no emission between 400 and 600 nm when excited at 340 nm. [c,d] See footnotes *c* and *d* in Table II.

$CHCl_3$–MeOH–EtOAc (9:0.8:0.2). Elution of the upper uv-absorbing zone and crystallization of the material from EtOAc gave 116 mg (66%) which after recrystallizations from EtOAc–MeOH and EtOAc had mp 198–201°; $[\alpha]^{20}D$ +53.2° (C_2H_5OH). *Anal.* ($C_{31}H_{38}N_4O_3$) N.

Results

The effects of various analogs of strophanthidin (**1**) on the Na^+- and K^+-activated ATPase from *Electrophorus* electric organ are presented in Table I. The 3-esters of strophanthidin are all potent inhibitors. The 3-salicylate (**2**) is strongly fluorescent. The 19-hydrazones, by contrast, are relatively inactive as inhibitors. The 19-salicylhydrazone (**3**) is strongly fluorescent. Either of the salicyl compounds might, therefore, serve as a Na^+- and K^+-ATPase affinity-directed fluorescent label for studies on purification of this enzyme.

The effect of environment on the fluorescence of strophanthidin 3-salicylate and strophanthidin 19-salicylhydrazone is shown in Tables II and III. The former compound fluoresced at 385 nm in water and at 340 nm in a less polar solvent (hexane–ethanol), while the latter compound fluoresced at 430 nm in water and at 464 nm in a less polar solvent (benzene–methanol). In the presence of increasing amounts of Na^+- and K^+-ATPase, the fluorescence of the 3-salicylate gradually shifted from that observed in aqueous



1, R′ = H; R = O

2, R′ = CC_6H_4OH-*o*; R = O

3, R′ = H; R = $NNHCC_6H_4OH$-*o*

solution to that observed in a nonpolar solvent. Thus, binding of the strophanthidin 3-salicylate to the Na^+- and K^+-ATPase preparation apparently occurs so that the fluorescent moiety is in a relatively nonpolar environment. By contrast, the fluorescence of the 19-salicylhydrazone is not affected by the presence of the enzyme preparation. This may reflect either a much weaker binding of this compound to the enzyme preparation, in line with its weaker inhibitory activity, or the fluorescent moiety may remain in an aqueous environment after binding of the compound. It is of interest that the fluorescence of the 19-salicylhydrazone appears somewhat decreased in the presence of the Na^+- and K^+-ATPase preparation (Table III), while the converse has been reported for the 19-sulfonaphthylhydrazone of hellebrigenin on interaction with enzyme from beef brain.[11] Further study will be necessary to determine if the 3-salicylate does bind selectively to the active site of Na^+- and K^+-ATPase and if it can be used for following purification of this enzyme. The preliminary results reported here suggest that the 3 position is a favorable location for introducing a fluorescent or spin-label probe[16] into a cardioactive steroid.

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