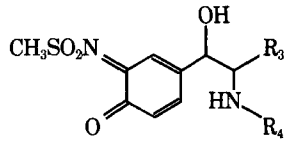
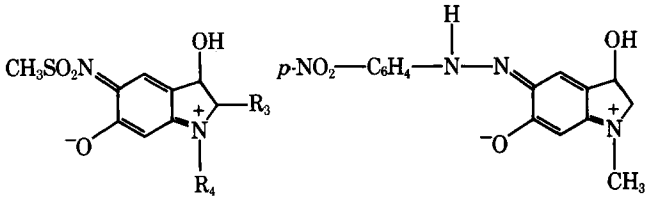


VII



VIII IX

That V, an isomer of I differing only in the positions of the ring substituents, produced only about one-twentieth the fluorescence of I was somewhat surprising. No explanation is offered, but it is interesting to note that compounds in this series (*i.e.*, those with the methanesulfonamido group *para* to the ethanolamine side chain) also have considerably lower adrenergic potency (4).

Prasad *et al.* (2) showed that isoproterenol and isoproterenolsulfonic acid do not interfere with their fluorometric method. Similarly, the ketone VI and the sulfonic acid IV do not form fluorescent species under the conditions used to analyze I. These final tests were designed only to demonstrate the lack of interference from IV and VI and do not preclude the possibility that these compounds might form fluorescent derivatives under different conditions.

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* Present address: Armak Co., Chicago, Ill.

‡ To whom inquiries should be directed.

Thiocardenolides I: Synthesis and Biological Actions of 3β-Thiocyanato-14β-hydroxy-5β-card-20(22)-enolide

HANLEY N. ABRAMSON *‡, CHIAN L. HUANG *, THOMAS F. WU *, and THOMAS TOBIN ‡

Abstract □ The synthesis of a 3β-thiocyanatocardenolide is described. The compound exhibited about 0.1 times the cardiotonic effect of digitoxigenin in the isolated frog heart preparation. At a dosage of 20 mg/kg in the intact rat, it elicited ECG changes similar to those seen with a 10-mg/kg dose of digitoxigenin. Studies also revealed the new cardenolide to be a reversible inhibitor of sodium- and potassium-activated adenosine triphosphatase.

Keyphrases □ Thiocardenolides—3β-thiocyanato-14β-hydroxy-5β-card-20(22)-enolide synthesized, screened for cardiotonic activity and effect on sodium- and potassium-activated adenosine triphosphatase □ Cardiotonic agents, potential—3β-thiocyanato-14β-hydroxy-5β-card-20(22)-enolide synthesized and screened □ Adenosine triphosphatase, sodium and potassium activated—effect of 3β-thiocyanato-14β-hydroxy-5β-card-20(22)-enolide evaluated

Most naturally occurring cardioactive steroids are characterized by the presence of a free hydroxyl group at position 3β, as in digitoxigenin (I). While the changes in biological properties elicited by glycosidation, esterification, epimerization, and oxidation of the cardenolide 3β-hydroxyl group are well known and have been reviewed (1), nothing has been published concerning the effect of replacing the oxygen atom at position 3β by other heteroatoms such as nitrogen or sulfur.

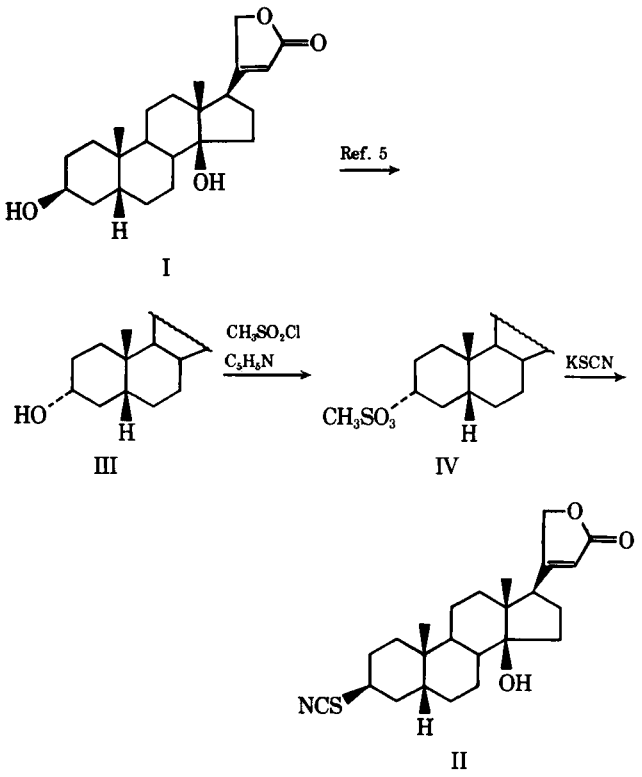
The 3α- and 3β-aminodigitoxigenins were prepared (2), but their pharmacological actions were not described. Removal of the 3β-oxygen atom of I to give 3-deoxydigitoxigenin results in a compound that has cardiotonic activity on the isolated frog heart comparable to that of I (3, 4).

As part of a program to prepare and study the pharmacological actions of sulfur-containing cardenolides, the synthesis and biological properties of the 3β-thiocyanato analog (II) of I are now reported.

EXPERIMENTAL[1]

Chemistry—3β-Thiocyanato-14β-hydroxy-5β-card-20(22)-enolide (II) was prepared by a two-step sequence from 3-epidigitoxi-

[1] Melting points were taken on a Fisher-Johns melting-point stage and are uncorrected. UV absorption spectra were determined in 95% ethanol on a Beckman model DK2A recording spectrophotometer. IR absorption spectra were recorded on a Beckman model 8 recording spectrophotometer. NMR spectra were determined on a Varian EM 360 spectrometer. Microanalyses were conducted by Spang Microanalytical Laboratory, Ann Arbor, Mich. TLC was carried out with silica gel G. Column chromatography was carried out with silica gel 60 (Brinkmann 7734).



Scheme I

genin (III) (Scheme I). While the methanesulfonate ester (IV) of III was readily prepared, attempts to convert III to its *p*-toluenesulfonate ester, as described by Sawlewicz *et al.* (2), gave only starting material.

3α-Methanesulfonyloxy- 14β-hydroxy-5β-card-20(22) -enolide (IV)—A solution of 545 mg (1.46 mmoles) of III (5) in 10 ml of dry pyridine was cooled to 0° in an ice–salt bath and treated with 3.6 ml of freshly distilled methanesulfonyl chloride. The mixture was placed in the refrigerator for 3 hr. TLC [benzene–ethyl acetate (1:2)] showed the reaction to be complete. The reaction mixture then was poured onto ice, and the precipitate was collected by filtration, washed with water, and dried *in vacuo*.

The crude product weighed 617 mg (93% yield), mp 134–138°, and was suitable for use in the next reaction; IR: ν_{max} (chloroform) 3620 (OH), 1790 and 1745 (butenolide doublet), 1630 (C=C), and 1360 and 1170 (sulfonate doublet) cm^{-1}; UV: λ_{max} (ethanol) 217 nm (ϵ = 19,500); NMR ($CDCl_3$; tetramethylsilane as internal standard): δ 0.88 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 3.10 (3H, s, SO_2CH_3), 4.91 (2H, s, 21-CH_2), and 5.88 (1H, s, 22=CH—).

3β-Thiocyanato-14β-hydroxy-5β-card-20(22)-enolide (II)—A solution of IV (500 mg, 1.10 mmoles) and 250 mg (2.59 mmoles) of potassium thiocyanate in 6 ml of dimethylformamide was stirred at 100° for 2 hr. The reaction mixture was poured onto ice. The precipitate was filtered, washed with water, and dried *in vacuo*. The product was purified by column chromatography, using increasing concentrations of chloroform in benzene as the eluent. A total of 328 mg (72% yield) of pure II was eluted with chloroform–benzene (4:1). Recrystallization from acetone–water gave 229 mg, mp 234–234.5°; IR: ν_{max} (mineral oil) 3495 (OH), 2145 (sharp and medium, —SCN), 1790 and 1745 (butenolide doublet), and 1630 (C=C) cm^{-1}; UV: λ_{max} (ethanol) 216.5 nm (ϵ = 19,700); NMR (CD_3SOCD_3; tetramethylsilane as internal standard): δ 0.77 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 4.15 (1H, m, 3α-H), 4.90 (2H, s, 21-CH_2), and 5.94 (1H, s, 22=CH—).

Anal.—Calc. for $C_{24}H_{33}NO_3S$: C, 69.36; H, 8.00; N, 3.37; S, 7.72. Found: C, 69.37; H, 8.06; N, 3.42; S, 7.67.

While the thiocyanate anion may be considered to be an ambident nucleophile, the product II was identified as a thiocyanate rather than as an isothiocyanate on the basis of the IR spectrum. Thiocyanates show medium to strong, sharp absorption at 2170–2135 cm^{-1}, while isothiocyanates exhibit a very strong and broad band at 2150–2050 cm^{-1} (6). The multiplet centered at δ 4.15 in the NMR of II due to the deshielded equatorially oriented 3α-proton compares well with the value of δ 4.04 found for the equatorially oriented 3β-proton of 3α-thiocyanato-5α-steroids (7). Axial orientation of the proton at position 3 of II would be expected to result in absorption near δ 3.15 (7).

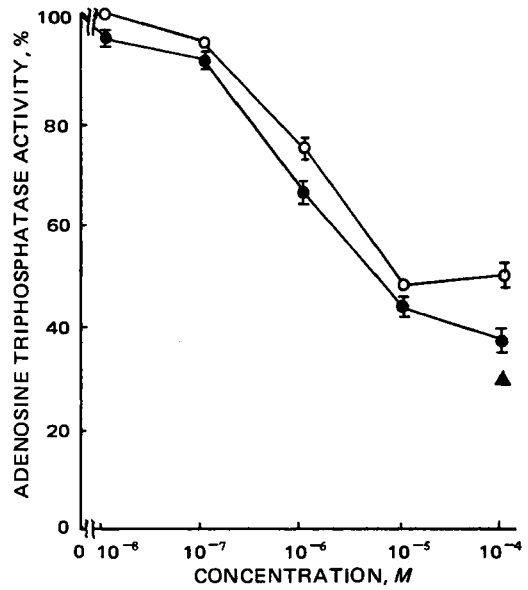


Figure 1—*Inhibition of a rat brain sodium- and potassium-activated adenosine triphosphatase preparation (10) (40 μg) in the presence of sodium (100 mmoles), potassium (15 mmoles), and adenosine triphosphate (5 mmoles) after incubation for 10 min at 37°. The average adenosine triphosphatase activity is 117 ± 4.0 μmoles P_i/mg protein/hr. Key: ○, inhibitory activity of II (means ± SE of determinations on six enzyme preparations); ●, inhibitory activity of I (means ± SE of determinations on four enzyme preparations); and ▲, inhibition by 10^{-4} M ouabain (mean of two determinations).*

Biological Evaluation—Isolated frog hearts (six preparations) were used to evaluate the cardiotonic effect of II according to Straub's method (8). Saline was used as the control, and digitoxigenin (I) was used as the reference standard. The frog hearts were perfused with Frog–Ringers solutions of both compounds, and the changes in chronotropic and inotropic responses were recorded on a smoked-drum chymograph. Analytically pure samples of II were used in the succeeding experiments.

The effect of II on the intact rat heart was studied using three animals. The animals were anesthetized with pentobarbital (45 mg/kg ip), and the cardiac actions were recorded on a polygraph recorder[2]. Saline was used as the control, and digitoxigenin was used as the standard.

All studies of sodium- and potassium-activated adenosine triphosphatase inhibition utilized the standard rat brain enzyme preparation (9) as modified by Tobin *et al.* (10). About 40 μg of the enzyme was incubated with 100 mmoles of sodium (Na^+), 15 mmoles of potassium (K^+), and 5 mmoles of adenosine triphosphate[3] for 10 min at 37°. The adenosine triphosphatase activity was measured and expressed as a percentage of the total enzyme activity [117 ± 4.0 μmoles of inorganic phosphate (P_i)/mg of protein/hr] at various concentrations of I and II. The results are presented in Fig. 1. The extent of inhibition by 10^{-4} *M* ouabain is indicated for comparison.

The effect of II on the equilibrium level of ^{3}H-ouabain binding to sodium- and potassium-activated adenosine triphosphatase was measured upon incubation of about 100 μg of the rat brain enzyme with 100 mmoles of sodium (Na^+), 0.2 mmole of magnesium (Mg^{+2}), and 5 mmoles of adenosine triphosphate at 37°. A tracer amount (3×10^{-10} mole) of ^{3}H-ouabain[4] (11.7 Ci/mmole) was added to the system at zero time. The counts per minute bound to

[2] Sanborn model 964.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] New England Nuclear, Boston, Mass.

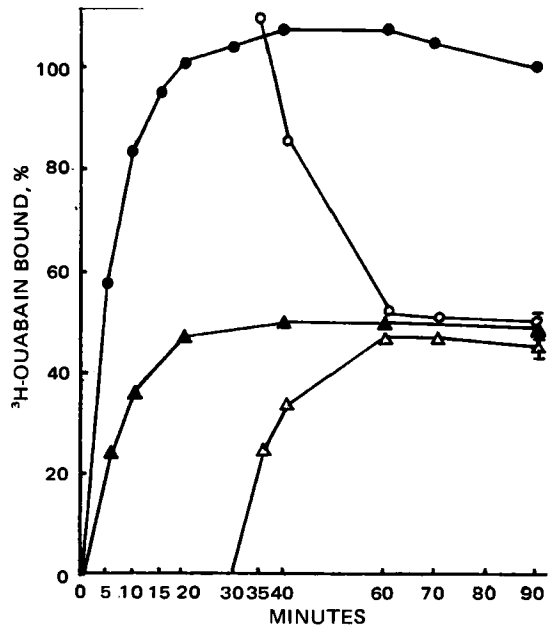


Figure 2—*Effect of II on the equilibrium level of ^{3}H-ouabain binding to a rat brain sodium- and potassium-activated adenosine triphosphatase preparation (10) (100 μg) in the presence of sodium (100 mmoles), magnesium (0.2 mmole), and adenosine triphosphate (5 mmoles) at 37°. Counts per minute of ^{3}H-ouabain at each time point is expressed as a percentage of the counts per minute of ^{3}H-ouabain bound at 90 min (equivalent to 11.4 ± 0.2 pmoles of ^{3}H-ouabain/mg of protein bound). All points are the means of experimental determinations on two different enzyme preparations, except for the 90-min data points which are the means ± SE of experimental determinations on four different enzyme preparations. Key: ●, control, 3 × 10^{-10} mole of ^{3}H-ouabain added at time zero; ▲, II (1 × 10^{-6} mole) and ^{3}H-ouabain added simultaneously; ○, II added 30 min after ^{3}H-ouabain; and △, ^{3}H-ouabain added 30 min after II.*

the enzyme was measured when: (*a*) ^{3}H-ouabain was added alone, (*b*) II was added to the binding system 30 min after the ^{3}H-ouabain, (*c*) II and ^{3}H-ouabain were added simultaneously, and (*d*) ^{3}H-ouabain was added 30 min after II. The results are summarized in Fig. 2. The number of counts per minute bound at each time point is expressed as a percentage of the number bound at 90 min in the presence of sodium ion and adenosine triphosphate, which was equivalent to 11.4 ± 0.2 pmoles of ^{3}H-ouabain/mg of protein bound.

In a study of the reversibility of the inhibition by II of sodium- and potassium-activated adenosine triphosphatase, about 400 μg of the enzyme was incubated with 100 mmoles of sodium chloride, 1 mmole of magnesium chloride, and 50 mmoles of tromethamine[3] (pH 7.4). The experiment was started by adding 0.1 mmole of adenosine triphosphate and 0.1 mmole of I or II or an equivalent volume of tromethamine buffer (control) to the system and incubating at 37° for 10 min.

At the end of this period, the enzyme was washed free of unreacted compound by twice centrifuging at 35,000×*g* and resuspending in 2 ml of tromethamine buffer. An aliquot was taken for protein estimation, and the enzyme was incubated at 37°. The adenosine triphosphatase activity of these preparations was expressed as a percentage of that of enzymes preincubated and washed in the absence of drugs, which averaged 152.6 ± 3.0 μmoles P_i/mg protein/hr. The results are shown in Fig. 3.

RESULTS AND DISCUSSION

In the isolated frog heart, no remarkable effect was seen in the dilution range of 1 × 10^{-5}–1 × 10^{-7} of II. At 1 × 10^{-4} dilution of II, the hearts responded with increased amplitude following a transient depressant response. A similar effect was observed with digitoxigenin at a dilution of 1 × 10^{-5}, except that no initial transient

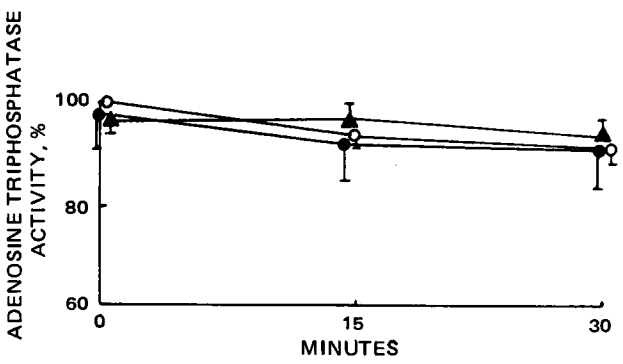


Figure 3—*Reversibility of inhibition of a rat brain sodium- and potassium-activated adenosine triphosphatase preparation (10) (400 μg) after incubation at 37° for 10 min in the presence of sodium (100 mmoles), magnesium (1 mmole), tromethamine buffer (50 mmoles, pH 7.4), adenosine triphosphate (0.1 mmole), and either I or II. Each time point represents the extent of adenosine triphosphatase activity (±SE of experimental determinations on four different enzyme preparations) after the enzyme has been washed free of either I or II and again incubated at 37°. Adenosine triphosphatase activity is expressed as a percentage of that of the enzymes preincubated and washed in the absence of drug, which averaged 152.6 ± 3.0 μmoles P_i/mg protein/hr. Key: ○, control (tromethamine buffer); ●, after incubating with 0.1 mmole of I and washing; and ▲, after incubating with 0.1 mmole of II and washing.*

depressant action was observed. At a dilution of 1 × 10^{-3}, II caused a decreased amplitude on the heart, which was not modified by atropine. Digitoxigenin exhibited the same response at a dilution of 1 × 10^{-4}. It is concluded that II has a stimulant action on the isolated frog heart at 1 × 10^{-4} dilution but a depressant effect at 1 × 10^{-3} dilution. The effect of II is thus about 0.1 that of digitoxigenin on the isolated frog heart.

Injection of 10 mg/kg iv of II had no noticeable effect on the lead II ECG of the intact rat heart. At a dosage level of 20 mg/kg, there was a decrease in the amplitude of the T wave and a reduction of the amplitude of the QRS complex. In general, the PR interval was prolonged with a shortened QT segment at 30 mg/kg. Similar responses were observed with digitoxigenin at lower doses (10 mg/kg).

The results of the interaction of II with sodium- and potassium-activated adenosine triphosphatase indicate that the inhibitory effect of II on the enzyme is slightly less than that of digitoxigenin (Fig. 1) and is reversible (Figs. 2 and 3). Figure 2 shows that the same equilibrium level of ^{3}H-ouabain was found regardless of the order of addition of the ligands, suggesting that II competes with the ouabain for the cardiotonic steroid binding site on the enzyme.

It thus appears that substitution of the 3β-hydroxyl group of digitoxigenin by a 3β-thiocyanato group does not seriously alter the ability of the cardenolide to interact at the steroid binding site of sodium- and potassium-activated adenosine triphosphatase, although there is a reduction in cardioactivity. This work is currently being expanded to a study of other thiocardenolides containing thiol and thioacetyl groupings at position 3β to ascertain whether it is possible to develop active-site-directed irreversible inhibitors of sodium- and potassium-activated adenosine triphosphatase that can act by the formation of disulfide bonds to the enzyme.

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ˣ To whom inquiries should be directed.

Fluorometric Determination of Cephradine in Plasma

ANTHONY F. HEALDˣ, CALLIXTUS E. ITA, and ERIC C. SCHREIBER

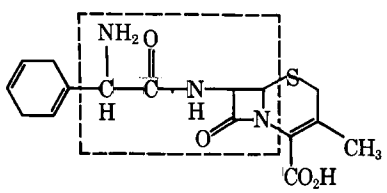
Abstract □ A fluorometric method was developed for the determination of cephradine in plasma. A fluorescent product is formed when samples of deproteinized plasma containing cephradine are heated for 3 hr at 100° and pH 1. The fluorescence is determined in sodium hydroxide solution (pH 13.5) at excitation and emission wavelengths of 350 and 445 nm, respectively. Only 0.1 ml of plasma is required, and concentrations of cephradine as small as 0.1 μg/ml may be determined. In plasma samples from a dog taken over a 10-hr period after an intramuscular injection of 250 mg of cephradine, essentially similar concentrations of cephradine were obtained by the fluorometric method and a standard microbiological bioassay.

Keyphrases □ Cephradine—fluorometric analysis, compared to microbiological bioassay, plasma □ Fluorometry—analysis, cephradine in plasma □ Antibiotics—cephradine, fluorometric analysis, plasma

Cephradine (I), 7-[D-2-amino-2-(1,4-cyclohexadien-1-yl)acetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, is a semisynthetic cephalosporin (1). The concentration in biological fluids of cephradine, a poorly bound antibiotic, may be determined microbiologically (1). Colorimetric assays for cephalosporins (2) and cephradine (3) also have been reported.

During studies of the bioavailability of cephradine, it became desirable to develop a rapid, sensitive, chemical assay of the concentration of this antibiotic in plasma. Jusko (4) described a fluorometric assay for ampicillin that appeared to be specific for penicillins containing the structure enclosed by the dotted line in Structure I.

During initial attempts to adapt this assay to cephradine, plasma concentrations of cephradine less than 5–10 μg/ml could not be determined. Because the first step in the formation of a fluorescent product from ampicillin is the acid hydrolysis of the β-lactam ring, the greater stability of this ring in cephalosporins might be a major factor in the reduced sensitivity of the assay when applied to cephradine. The present sensitive analytical method for cephradine was developed by increasing the severity of the hydrolysis conditions.



I

EXPERIMENTAL

Reagents—The following reagents were used: 10% aqueous trichloroacetic acid solution, 4 *N* sodium hydroxide solution, and 0.2 *M* potassium chloride–hydrochloric acid buffer, pH 1.0. Glass-distilled water was used throughout, and the use of polyethylene containers was avoided (5).

Cephradine Standard Curve—Cephradine standards were added to control plasma. Because of possible variations in the water content of separate batches of cephradine, each cephradine standard was calibrated microbiologically. All weights were expressed as micrograms or milligrams of microbiological activity. Aliquots of a stock solution of cephradine in water (100 μg/ml) were diluted with plasma to prepare a suitable range of working standards as well as a blank. Three 0.1-ml aliquots of each standard were carried through the procedure with each batch of plasma samples.

Microbiological Method—The concentration of cephradine in plasma samples was determined microbiologically by an agar-diffusion method, with *Sarcina lutea* (ATCC 9341) as the test organism.

Animal Experiment—Cephradine for injection (250 mg) was injected into the semitendinosus muscle of a male beagle dog (10.6 kg). Plasma was obtained at intervals for 10 hr, and the cephradine content of each sample was determined by both the spectrofluorometric and microbiological methods.

Fluorometric Assay Procedure—Fluorescence was measured[1] at excitation and emission wavelengths of 350 and 445 nm, respectively. The instrument was zeroed with a reagent blank and set to read 90% of full-scale deflection against a suitable cephradine standard, depending on the range of concentrations expected in the unknowns.

[1] Farrand Mark I spectrofluorometer.