Pergamon

Tetrahedron 56 (2000) 9625–9632

TETRAHEDRON

A Novel Cardenolide Photoaffinity Label for the Na/K-ATPase

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This paper is dedicated with great admiration and graditude to Professor Koji Nakanishi on the occasion of his 75th birthday

Received 7 September 2000; accepted 29 September 2000

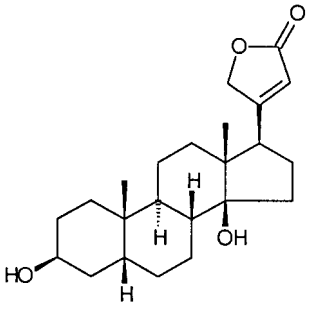
Abstract—A benzoylbenzoyloxy derivative of digitoxigenin has been prepared which is the first cardenolide with an affinity label in the lactone ring. It inhibits the Na^+/K^+-ATPase almost as effectively as digitoxigenin itself. The activity was lost when in addition a biotin tag was attached to ring A.

Introduction

The Na^+/K^+-ATPase (Na^+ pump) is a dimeric ($\alpha\beta$) transmembrane complex that is expressed in virtually all animal cells. It couples ATP hydrolysis with the export of Na^+ and the import of K^+. The ion gradients thus established over the membrane drive numerous co- and countertransporters that supply the cells inter alia with glucose and amino acids, regulate cell volume, Ca^{2+} concentration, and underlie nearly all electrical activity in the peripheral and central nervous system and in cardiac and skeletal muscle.[1–3] In addition, the Na^+/K^+-ATPase is the receptor for the cardioactive steroids. These compounds specifically inhibit the Na^+ pump, thereby raising the intracellular Na^+ concentration. The resulting decrease in the Na^+ gradient across the cell membrane of cardiac myocytes reduces the energy available for transport of Ca^{2+} out of the cell by Na^+/Ca^{2+} exchange. This ultimately leads to the (medicinally used) positive inotropic effect of these substances.[4]

The ATPase consists of a large catalytic α subunit and a smaller β subunit the function of which is still not completely understood. The complete amino-acid sequences of α subunits of various isoforms have been determined.[5] The protein chain spans the plasma membrane ten times (H1–H10 spans).

Mechanistic understanding both of the normal function of the enzyme and of the inhibition by the cardioactive steroids would demand knowledge of the three-dimensional structure of the protein and of the conformational intermediates in the catalytic cycle which is not available. All what is known today has been extracted from indirect informations such as twodimensional crystals, site-directed mutagenesis, fluorescence methods, affinity labeling, competition experiments with antibodies, structure-activity relationships (as far as the cardiotonic steroids are involved) and molecular modeling.[6–9]



digitoxigenin (**1**)

According to the present knowledge the major cytoplasmic loop of the α subunit contains the ATP binding and the phosphorylation sites. The cation binding sites are provided by amino acid residues of the intramembrane segments of the α unit. The cardioactive steroids (see digitoxigenin (**1**) as an example) interact with the extracellular portion. Both from site-directed mutagenesis and from affinity labeling experiments it has been inferred that the H1–H2 extracellular region is involved in binding. There are other labeling experiments that indicate that the H3–H4 region is involved in binding the lactone moiety.[10] These results

Keywords: cardenolides; Na,K-ATPase; affinity label; benzophenone.
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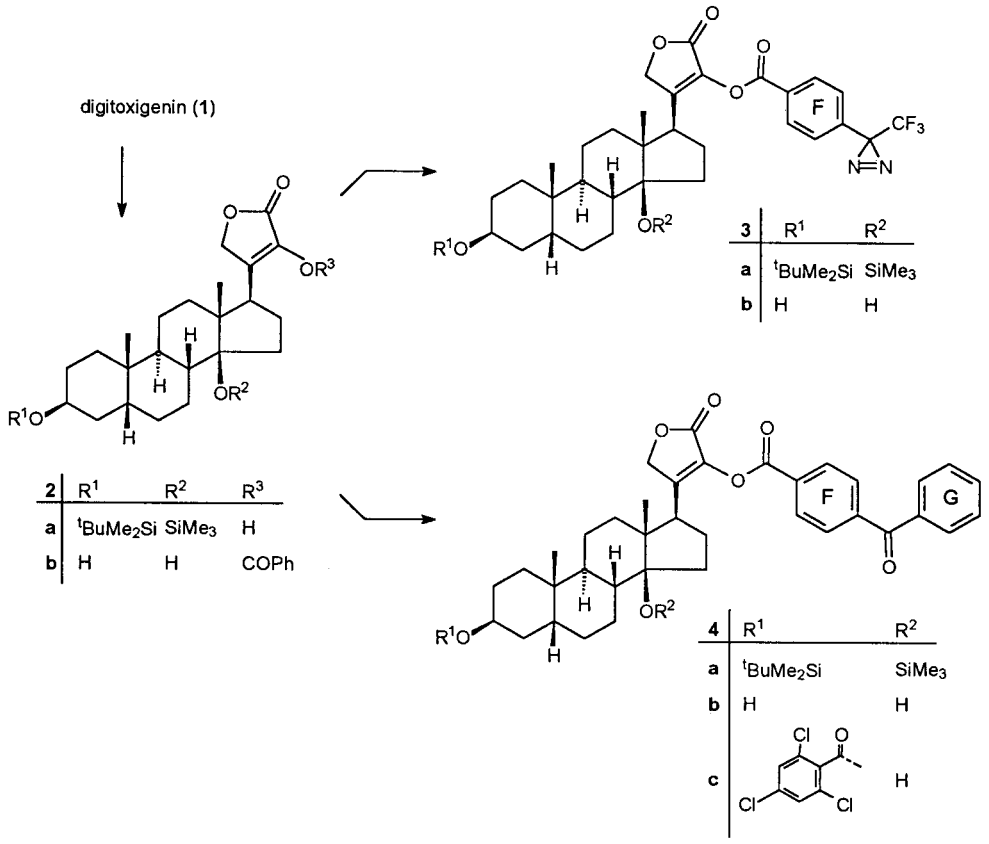
0040–4020/00/$ - see front matter
PII: S0040-4020(00)00916-9



Scheme 1.

have been summarized by Höltje and Anzali in a binding model[9] which, however, has been shown to be unable to explain all the experimental results.[11,12]

New results point to the major involvement of the H5–H6 domain in the inhibition of the enzyme by cardiotonic steroids (ouabain).[13,14]

With respect to affinity labeling, most labeling experiments have identified binding sites in the H1–H2 region.[15] In all cases the affinity label was in some way attached to ring A of the steroid. The only labeling experiment we are aware of, in which the affinity label was part of the side chain at C-17 was reported by McParland and coworkers who used a tritiated lactone analogue with a 24-azido-21-nor-20(22)-en-23-one side chain. Degradation of the labeled protein and sequence analysis showed the label to be in the extracellular domain between the H3 and H4 hydrophobic domains.[10]

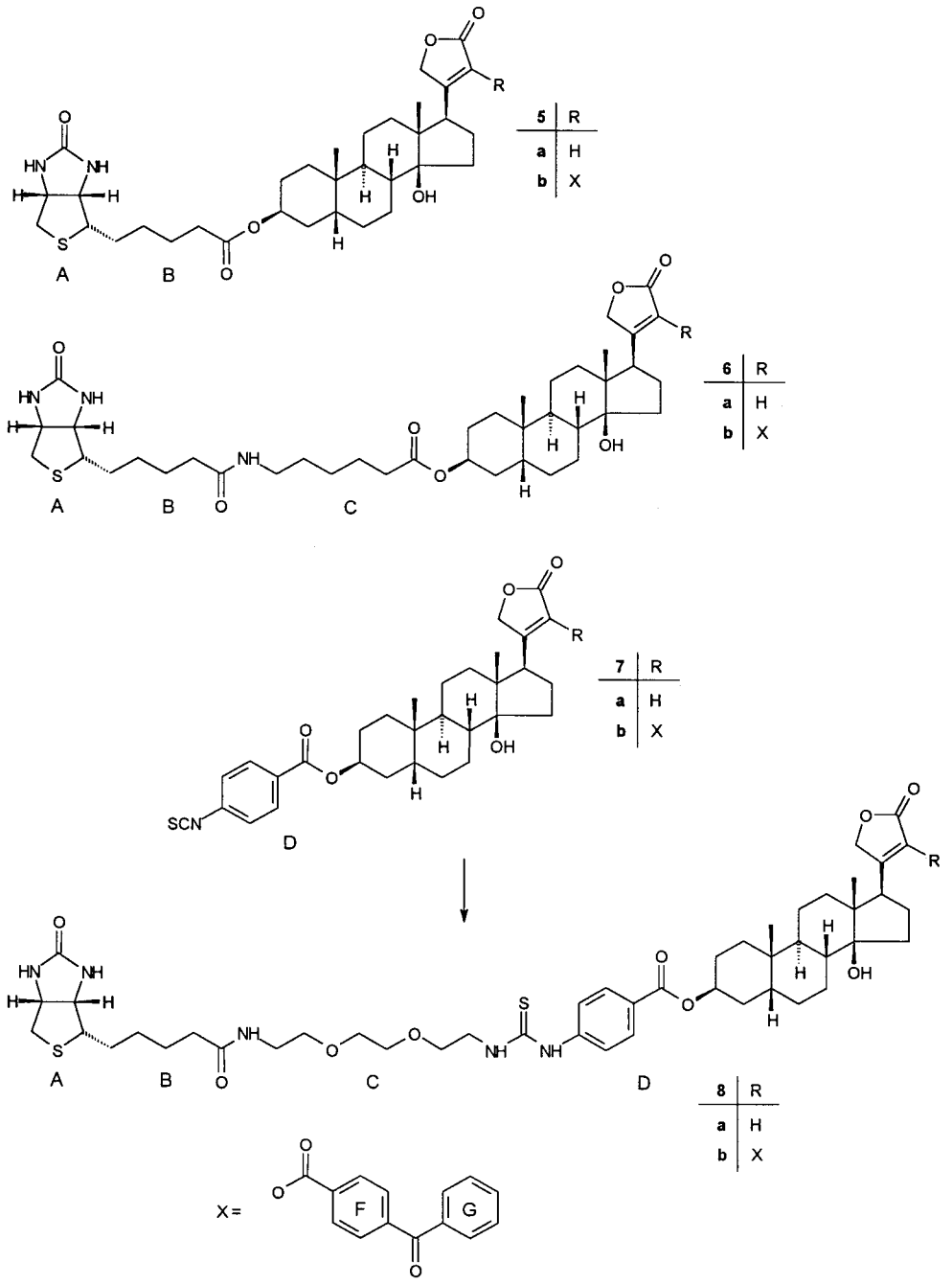
From many structure-activity studies it is quite clear, that the lactone side chain of the cardiotonic steroids is a main structural prerequisite for their interaction with the Na^+/K^+-ATPase.[7] It appeared to us, therefore, worthwhile to develop affinity labels with the reactive groups attached to the lactone ring. These studies will be detailed below.

Results and Discussion

Our work started from the observation, that digitoxigenin derivatives having an acetate and a benzoate group, respectively, attached to C-22 (see, for example formula **2b**) inhibit the Na^+/K^+-ATPase quite effectively.[11,12] It seemed promising, to study derivatives of **2b** with reactive groups attached to the aromatic ring (Scheme 1).

With this in mind we prepared compound **3b**. The aryl trifluoromethyl azirine is known to decompose to a carbene on irradiation at 350 nm.[16] Thus, the known derivative **2a** of 22-hydroxy-digitoxigenin[11] was esterified with 4-(3-trifluoromethyl-3-*H*-diazirinyl)benzoic acid[17] using dicyclohexylcarbodiimide in the presence of Steglich's base[18] to give **3a** in 84% yield. Removal of the silyl protecting groups was achieved by acid treatment in methanolic solution.[11] Unfortunately, **3b** did not at all inhibit the Na^+/K^+-ATPase (see Table 1).

The next compound to be studied was the benzoylbenzoate **4a**. Again, the DCC method was employed, and the yield was 90%. Deprotection under the conditions described above provided **4a**, but to a major extent the dimethyl acetal at the benzophenone unit (formula not shown) was formed.[19] The unwanted side product was omitted when after the cleavage in methanolic solution the crude reaction product was treated with aqueous acid.[20] The overall yield was 94%. **4b** turned out to inhibit the Na^+/K^+-ATPase very effectively (see Table 1). This is an exciting result since for the first time an active inhibitor of the Na^+/K^+-ATPase was found with the photo-reactive group in the lactone ring. In addition, it can be concluded that in the binding pocket around the lactone ring there must be even more space



Scheme 2.

than previously assumed.[11] It should be remembered in this context that obviously the ester group is involved in binding to the enzyme since many other 22-substituted digitoxigenin derivatives have been shown to be inactive.[11]

Usually, after photolabeling the enzyme is digested enzymatically and the labeled fragments are isolated and submitted to structural analysis. Biotin-tagging is a powerful method for identifying labeled protein fragments and isolating them by affinity chromatography based on the strong interaction of biotin with either avidin or streptavidin.[21]

We attempted, therefore, to add a biotin tag to the oxygen at C-3 of **4b**. In a first series of experiments (Scheme 2) it was tried to prepare compound **5b**. Unfortunately, attempted esterification of **4b** with biotin using the DCC method was unsuccessful. Then it was tried to use the *N*-hydroxy succinimide derivative of biotin as well as the acid chloride[22] as acylating reagents, again without success. The Staab method failed, too.[23] The same results were obtained when digitoxigenin was employed as a model compound. Finally after much experimentation it was shown that the Yamaguchi method[24] provided **5a** in 54% yield. But even this method did not reliably provide **5b**. In one experiment a yield of 35% was obtained but many other experiments failed. Instead the trichlorobenzoate **4c** was isolated.

Table 1. $K_{0.5}$ values for Na^+/K^+ pump inhibition by digitoxigenin and derivatives **2b**, **3b**, **4b**, **5a**, **5b**, **8a**, **8b** in cardiac cells (The $K_{0.5}$ value represents the drug concentration required for half-maximal Na^+/K^+ pump inhibition. The pump exchanges three intracellular Na^+ for two extracellular K^+ per ATP molecule split and generates thereby the pump current I_p. This current was measured as an indicator of pump activity in isolated guinea pig cardiac ventricular cells or sheep cardiac Purkinje cells (from the conducting system) by means of whole-cell recording.[25] The measurements were performed in a bathing solution including 144 mM NaCl and 5.4 mM KCl. The patch pipette solution contained inter alia 50 mM NaCl and 110 mM CsCl. pH was 7.4 in both media. The compounds were added to the external solution from 10^{-2} M ethanolic or DMSO stock solutions and their inhibitory effect on I_p was measured. The final concentrations of the solvents had no effect on I_p. The experiments were carried out at 32°C and 0 mV membrane (holding) potential. Note the higher sensitivity of the Purkinje cells to cardiac steroids. 'n' indicates the number of measurements.)

Compound	Guinea-pig ventricular cells $K_{0.5}$ [M]	*n*	Sheep cardiac Purkinje cells $K_{0.5}$ [M]	*n*
Digitoxigenin (1)	3.0×10^{-6}	50	1.9×10^{-7}	8
2b	–		4.1×10^{-6}	17
3b	5.0×10^{-5}	2		
4b	1.2×10^{-5}	28	6.0×10^{-7}	8
5a	1.0×10^{-6}	4		
5b	1.4×10^{-5}	4		
8a	8.0×10^{-6}	11		
8b	No effect	5		

5b only weakly inhibited the Na^+/K^+-ATPase. On the other hand, the biotinylated digitoxigenin **5a** was quite active. In order to find out whether a longer spacer arm would lead to better results, both digitoxigenin (**1**) and **4b** were converted into the biotinylated derivatives **6a** and **6b**, respectively. Again, the Yamaguchi method was only partly successful. The yields were only in the range of 10–14% and we could not prepare sufficient amounts for the biological tests. In a last set of experiments digitoxigenin (**1**) was converted into the isothiocyanatobenzoate **7a** on treatment with 4-isothiocyanatobenzoyl chloride. This reaction worked quite well and provided **7a** in 68% yield. **7a** was in turn converted into **8a** on treatment with the appropriate amine derivative of biotin. The yield of thiourea **8a** was 74%. In the same way **4b** was converted to **7b** (69%) in a very slow reaction and thence to the rather unstable compound **8b** (44%). **8b** is characterized by a nice ESI mass spectrum. The ^1H NMR spectra suffered from broad peaks obviously caused by restricted rotation in some parts of the molecule. Heating to 80°C improved the quality of the NMR spectra.

The inhibition of the Na^+/K^+ pump by both **8a** and **8b** were studied as summarized in Table 1. **8a** had a reduced activity whereas **8b** was completely inactive.

Obviously, there is a binding pocket for the cardioactive steroids which is accessible to both **4b** and **5a** with one extra substituent but not to **5b** that seems to be too big. In the same way the results for **8a** and **8b** can be explained.

From these results summarized in Table 1 it has to be concluded that the isolation of labeled peptides in affinity labeling experiments will not be possible making use of the biotin–avidin method. An alternative would be to identify labeled fragments of the Na^+/K^+-ATPase with antibodies directed against the steroid moiety. We used the ABBOTT AxSYM Digitoxin system which is based on a fluorescence polarisation immuno assay in which digitoxigenin and a fluorescein-labeled digitoxin derivative compete for the binding sites at the polyclonal antibodies (canin serum). It turned out that the biotinylated digitoxin derivative **5a** was identified by the antibodies at a concentration of 10^{-7} mol/l. In the control experiment digitoxigenin was recognized at a concentration of less than 10^{-7} mol/l. Unfortunately, **4b** was not recognized by the antibodies (see Table 2). This result probably means that the lactone part of the cardenolides interacts with the antibody as has been found for monoclonal anti-digoxin antibodies.[26]

Table 2. Recognition of compound **1**, **4b**, **5a** by anti-digitoxin antibodies

Compound	Sample concentration (M)	Result in digitoxin concentrations
1	10^{-8}	9.80 ng/ml (1.28×10^{-8} M)
4b	10^{-7}	No effect
5a	10^{-7}	61.76 ng/ml (8.07×10^{-8} M)

The result of our investigations is that neither the biotin–avidin/streptavidin technology nor affinity chromatography using anti-digitoxin antibodies can be used for the isolation of labeled fragments of the Na^+/K^+-ATPase. In any case, the compounds described here should be valuable tools for further work in this area. For the affinity labeling studies presumably recourse has to be made to analytical methods based on radioactive isotopes. Since the synthesis of *p*-benzoylbenzoic acid is very simply achieved, a radioactively labeled derivative should be easily accessible.[27]

Experimental

General

NMR: GEMINI 200 (Varian), GEMINI 2000 (Varian), GEMINI 300 (Varian), DRX 400 (Bruker); chemical shifts are given in δ values, CH_3, CH_2, CH groups and quaternary carbons when identified by APT are indicated by (−) (CH_3, CH) and (+) (CH_2, C_q), respectively. Mass spectrometry: FAB MS: VG Autospec (Fisons, 3-nitrobenzylalcohol matrix), ESI MS: FT-ICR-MS Apex II (Bruker Daltonics, acetonitrile–formic acid, positive ion mode). IR spectra: FT-IR ATI Mattson (Genesis Series, KBr). UV spectra: DU-650 (Beckman, acetonitrile).

22-[4-(3-Trifluoromethyl-3*H*-diazirinyl)benzoyloxy]-3β-(*tert*-butyldimethylsilyloxy)-14-trimethylsilyloxy-5β,14β-card-20(22)-enolide (3a). A solution of **2a** (100 mg; 173 μmol) and 4-dimethylaminopyridine (2.8 mg; 23 μmol) in dichloromethane (ca. 5 ml) was added to a mixture of *N*,*N*′-dicyclohexylcarbodiimide (71 mg; 346 μmol) and 4-(3-trifluoromethyl-3*H*-diazirinyl)benzoic acid (80 mg; 346 μmol). The reaction mixture was stirred at 20°C for 2 h (white precipitate) and then filtered. The filtrate was diluted with dichloromethane and the solution washed with water then with 5 percent sodium hydrogen carbonate. The aqueous phases were reextracted with dichloromethane. Drying of the combined organic phases with sodium sulfate, solvent evaporation and LC (petroleum

ether–ethyl acetate 12:1) provided **3a** (115 mg, 84%). IR (KBr): 1779, 1754, 1633, 1260, 1196, 1159, 1118, 1056, 837 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.01 (s, 6H, $^tBuSi(CH_3)_2$), 0.16 (s, 9H, $(CH_3)_3Si$), 0.87 (s, 9H, $(CH_3)_3C$), 0.90 and 0.92 (2 s, respectively 3H, CH_3-18, CH_3-19), 1.00–2.05 (om (overlapping multiplets)), 2.85–2.95 (m, 1H, 17α-H), 4.02 (broad s, 1H, 3α-H), 4.90+5.02 (AB, 2H, CH_2-21, $^2J_{AB}$=17.2 Hz), 7.30+8.15 (AA′BB′, 4H, arom. H, $^3J_{AB}$=8.5 Hz). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=−4.39 (−) ($^tBuSi(CH_3)_2$), 3.49 (−) ($Si(CH_3)_3$), 18.0 (−) (C-18), 18.6 (+) ($C(CH_3)_3$), 21.3 (+), 24.1 (+), 24.3 (−) (C-19), 25.8 (+), 26.3 (−) ($C(CH_3)_3$), 27.2 (+), 28.9 (+) (q, $C(CF_3)$, $^2J_{C,F}$=41.1 Hz), 29.0 (+), 30.3 (+), 34.4 (+), 34.9 (+), 36.2 (+), 36.3 (−) and 37.4 (−) (C-5, C-9), 41.3 (−) (C-8), 42.4 (+) (C-12), 48.3 (−) (C-17), 52.3 (+) (C-13), 67.6 (−) (C-3), 70.0 (+) (C-21), 91.8 (+) (C-14), 122.3 (+), (q, CF_3, $^1J_{C,F}$=275 Hz), 127.1 (−) (q, C-3^F, $^4J_{C,F}$=1.5 Hz), 129.6 (+) (C-1^F), 131.2 (−) (C-2^F), 135.2 (+) and 135.5 (+) (C-22, C-4^F), 154.8 (+) (C-20), 162.5 (+) (*C*OAr), 167.8 (+) (C-23). ^{19}F NMR (188.2 MHz, $CDCl_3$): δ=12.9 (s, CF_3). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=233 nm (19500). $C_{41}H_{59}F_3N_2O_6Si_2$ (789.10), FAB MS: *m/z* (%)=811 (5; $[M+Na]^+$), 789 (2; $[M+H]^+$), 567 (31; $[M+H-{}^tBuMe_2SiOH-Me_3SiOH]^+$), 213 (48; $[COC_6H_4CN_2CF_3]^+$), 185 (100; $[COC_6H_4CF{=}CF_2]^+$).

22-[4-(3-Trifluoromethyl-3*H*-diazirinyl)benzoyl]oxy-3β,14-dihydroxy-5β,14β-card-20(22)-enolide (3b). To **3a** (99 mg; 125 μmol) a solution of *p*-toluenesulfonic acid monohydrate (10 ml of a 10 mM solution in methanol) was added. The mixture was stirred at 20°C for 14 h. Usual work-up and LC (petroleum ether–ethyl acetate 2:3) furnished **3b** (72 mg, 95%). IR (KBr): 1756, 1632, 1344, 1264, 1230, 1197, 1160, 1121 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.94 (s, 6H, CH_3-18, CH_3-19), 1.15–2.20 (om), 2.88–2.97 (m, 1H, 17α-H), 4.01 (broad s, 1H, 3α-H), 4.94+5.20 (AB, 2H, CH_2-21, $^2J_{AB}$=17.8 Hz), 7.31+8.11 (AA′BB′, 4H, arom. H, $^3J_{AB}$=8.5 Hz). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=15.7 (−) (C-18), 21.5 (+), 21.7 (+), 24.1 (−) (C-19), 25.6 (+), 26.8 (+), 28.3 (+), 28.8 (+) (q, $C(CF_3)$, $^2J_{C,F}$=40.8 Hz), 30.0 (+), 33.6 (+), 33.7 (+), 35.8 (+), 35.9 (−) and 36.3 (−) (C-5, C-9), 40.3 (+) (C-12), 42.0 (−) (C-8), 47.6 (−) (C-17), 50.4 (+) (C-13), 67.2 (−) (C-3), 70.3 (+) (C-21), 85.8 (+) (C-14), 122.3 (+) (q, CF_3, $^1J_{C,F}$=275 Hz), 127.2 (−) (q, C-3^F, $^4J_{C,F}$=1.5 Hz), 129.6 (+) (C-1^F), 131.3 (−) (C-2^F), 135.4 (+) and 135.6 (+) (C-22, C-4^F), 155.5 (+) (C-20), 162.8 (+) (*C*OAr), 168.0 (+) (C-23). ^{19}F NMR (188.2 MHz, $CDCl_3$): δ=12.9 (s, CF_3). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=231 nm (20300). $C_{32}H_{37}F_3N_2O_6$ (602.65), FAB MS: *m/z* (%)=625 (5; $[M+Na]^+$), 603 (2; $[M+H]^+$), 585 (9; $[M+H-H_2O]^+$), 567 (7; $[M+H-2H_2O]^+$). HRMS: calcd for $C_{32}H_{38}F_3N_2O_6$ ($[M+H]^+$) 603.2682, found 603.2692.

22-(4-Benzoylbenzoyloxy)-3β-(*tert*-butyldimethylsilyloxy)-14-trimethylsilyloxy-5β,14β-card-20(22)-enolide (4a). A solution of **2a** (200.0 mg; 346 μmol) and **4**-dimethylaminopyridine (5.5 mg; 45 μmol) in dichloromethane (5 ml) was added to a mixture of *N*,*N*′-dicyclohexylcarbodiimide (128.7 mg; 624 μmol) and 4-benzoylbenzoic acid (141.2 mg; 624 μmol). The reaction mixture was stirred at 20°C for 4 h. A white precipitate was filtered off and the filtrate was worked up as usual. LC (petroleum ether–ethyl acetate 8:1→6:1) furnished 244 mg (90%) of **4a**. Mp 171–173 °C (from petroleum ether). IR (KBr): 1754, 1633, 1254, 1124, 1056, 837 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.01 (s, 6H, $^tBuSi(CH_3)_2$), 0.18 (s, 9H, $(CH_3)_3Si$), 0.88 (s, 9H, $(CH_3)_3C$), 0.91 (s, 3H, CH_3-19), 0.96 (s, 3H, CH_3-18), 1.05–2.10 (om), 2.90–3.01 (m, 1H, 17α-H), 4.03 (broad s, 1H, 3α-H), 4.93+5.04 (AB, 2H, CH_2-21, $^2J_{AB}$=17.0 Hz), 7.46–7.57 (m, 2H, arom. H_m, ring G), 7.59–7.69 (m, 1H, arom. H_p, ring G), 7.78–7.86 (m, 2H, arom. H_o, ring G), 7.89+8.25 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=8.4 Hz). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=−4.51 (−) and −4.49 (−) ($^tBuSi(CH_3)_2$), 3.41 (−) ($(CH_3)_3Si$), 17.9 (−) (C-18), 18.5 (+) ($C(CH_3)_3$), 21.2 (+), 24.0 (+), 24.2 (−) (C-19), 25.8 (+), 26.2 (−) ($C(CH_3)_3$), 27.1 (+), 28.9 (+), 30.2 (+), 34.4 (+), 34.8 (+), 36.1 (+), 36.2 (−) and 37.3 (−) (C-5, C-9), 41.2 (−) (C-8), 42.3 (+) (C-12), 48.2 (−) (C-17), 52.3 (+) (C-13), 67.6 (−) (C-3), 70.0 (+) (C-21), 91.8 (+) (C-14), 129.1 (−) (C-3^G), 130.4 (−) and 130.8 (−) (C-2^F, C-3^F), 130.6 (−) (C-2^G), 131.5 (+) and 137.2 (+) (C-1^F, C-1^G), 133.7 (−) (C-4^G), 135.3 (+) (C-22), 142.9 (+) (C-4^F), 155.0 (+) (C-20), 162.9 (+) (*C*OAr^F), 168.0 (+) (C-23), 196.3 (+) (*C*OAr^G). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=255 nm (18500). $C_{46}H_{64}O_7Si_2$ (785.18), FAB MS: *m/z* (%)=807 (4; $[M+Na]^+$), 727 (3; $[M+H-C_4H_{10}]^+$), 563 (8; $[M+H-{}^tBuMe_2SiOH-Me_3SiOH]^+$), 209 (100, $[C_6H_5COC_6H_4CO]^+$). HRMS: calcd for $C_{46}H_{64}O_7Si_2Na$ ($[M+Na]^+$) 807.4088, found 807.4090.

22-(4-Benzoylbenzoyloxy)-3β,14-dihydroxy-5β,14β-card-20(22)-enolide (4b). To **4a** (244 mg; 311 μmol) *p*-toluenesulfonic acid monohydrate (20 ml of a 10 mM solution in methanol) was added and the mixture was stirred at 20°C for 24 h. After usual work-up the residue was redissolved in 4:1 dioxane–water (15 ml). HCl (1 ml of a 0.02 M aqueous solution) was added and the mixture was stirred at 40°C for 6 h. After solvent evaporation, usual work-up and LC (petroleum ether–ethyl acetate=1:1→1:2) 175 mg (94%) of **4b** were obtained. IR (KBr): 1761, 1658, 1256, 1118, 1022, 710 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.83–1.01 (m) therein 0.94 and 0.96 (2 s, CH_3-18, CH_3-19), 1.15–2.22 (om), 2.93–3.02 (m, 1H, 17α-H), 4.11 (broad s, 1H, 3α-H), 4.96+5.22 (AB, 2H, CH_2-21, $^2J_{AB}$=17.8 Hz), 7.46–7.57 (m, 2H, arom. H_m, ring G), 7.59–7.69 (m, 1H, arom. H_p, ring G), 7.78–7.84 (m, 2H, arom. H_o, ring G), 7.88+8.25 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=8.6 Hz). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=15.9 (−) (C-18), 21.6 (+), 21.9 (+), 24.2 (−) (C-19), 25.7 (+), 26.9 (+), 28.4 (+), 30.1 (+), 33.7 (+), 33.8 (+), 35.9 (+), 36.0 (−) and 36.5 (−) (C-5, C-9), 40.4 (+) (C-12), 42.2 (−) (C-8), 47.7 (−) (C-17), 50.5 (+) (C-13), 67.3 (−) (C-3), 70.3 (+) (C-21), 85.8 (+) (C-14), 129.1 (−) (C-3^G), 130.4 (−) and 130.9 (−) (C-2^F, C-3^F), 130.7 (−) (C-2^G), 131.5 (+) and 135.4 (+) and 137.2 (+) (C-22, C-1^F, C-1^G), 133.7 (−) (C-4^G), 142.9 (+) (C-4^F), 155.4 (+) (C-20), 163.0 (+) (*C*OAr^F), 168.0 (+) (C-23), 196.3 (+) (*C*OAr^G). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=255.0 nm (25800). $C_{37}H_{42}O_7$ (598.74).

14-Hydroxy-3β-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyloxy]-5β,14β-card-20(22)-enolide (5a). To a solution of biotin (73.3 mg;

300 μmol) and triethylamine (45.5 mg; 450 μmol) in THF (ca. 6 ml, freshly distilled under Ar) 2,4,6-trichlorobenzoyl chloride (73.2 mg; 300 μmol) was added dropwise. The mixture was stirred at 20°C for 4 h. After solvent evaporation the residue was redissolved in toluene (5 ml) and a solution of digitoxigenin (74.9 mg; 200 μmol) and 4-(1-pyrrolidinyl)-pyridine (PPP, 59.3 mg; 400 μmol) in toluene (1 ml) was added. The reaction mixture was stirred at 40°C for 4 h and at 20°C for 16 h. Usual work-up and LC (ethyl acetate–methanol ∞→5:1) furnished 65.1 mg (54%) of **5a**. 11.0 mg (15%) of digitoxigenin were recovered. IR (KBr): 1739, 1706, 1453, 1263, 1151, 1024 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.85 (s, 3H, CH_3-18), 0.94 (s, 3H, CH_3-19), 1.10–2.22 (om), 2.31 (t, 2H, CH_2-2^B, $^3J^B_{2,3}$=7.3 Hz), 2.74+2.89 (AB (ABX), 2H, CH_2-6^A, $^2J_{AB}$=13.2 Hz, $^3J_{BX}$=4.8 Hz) therein 2.70–2.82 (m, 1H, 17α-H), 3.07–3.19 (m, 1H, CH-4^A), 4.24–4.33 (m, 1H, CH-$3a^A$), 4.44–4.53 (m, 1H, CH-$6a^A$), 4.80+4.99 (AB (ABX), 2H, CH_2-21, $^2J_{AB}$=18.2 Hz, $^4J_{AX}$=1.5 Hz), 5.05 (bs, 1H, 3α-H), 5.68 (broad s, 1H, NH), 5.85 (s, 1H, 22-H), 5.94 (broad s, 1H, NH). ^{13}C NMR (50.3 MHz, APT, HETCOR, $CDCl_3$): δ=16.3 (−) (C-18), 21.6 (+), 21.7 (+), 23.2 (+), 24.3 (−) (C-19), 25.4 (+) (C-3^B), 25.5 (+), 26.8 (+), 27.4 (+), 28.8 (+), 28.9 (+), 31.0 (+), 33.6 (+), 34.9 (+) (C-2^B), 35.6 (+), 36.1 (−) and 37.4 (−) (C-5, C-9), 40.4 (+) (C-12), 41.0 (+) (C-6^A), 42.2 (−) (C-8), 50.1 (+), 51.4 (−) (C-17), 56.0 (−) (C-4^A), 60.6 (−) (C-$6a^A$), 62.5 (−) (C-$3a^A$), 70.8 (−) (C-3), 74.0 (+) (C-21), 85.8 (+), 118.0 (−) (C-22), 164.3 (+) (C-2^A), 173.7 (+) (C-1^B), 175.1 (+) and 175.4 (+) (C-23, C-20). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=214.5 nm (11200). $C_{33}H_{48}N_2O_6S$ (600.82), FAB MS: *m/z* (%): 623 (9; $[M+Na]^+$), 601 (8; $[M+H]^+$), 583 (11; $[M+H-H_2O]^+$), 245 (58; $[biotin+H]^+$). HRMS: calcd for $C_{33}H_{49}N_2O_6S$ ($[M+H]^+$) 601.3311, found 601.3294.

22-(4-Benzoylbenzoyloxy)-14-hydroxy-3β-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-pentanoyloxy]-5β,14β-card-20(22)-enolide (5b). 4b (63.6 mg; 106 μmol) was converted into **5b** using the procedure described for **5a**. LC (ethyl acetate–methanol ∞→5:1) provided **5b** (31.0 mg, 35%). In addition, 23.5 mg (27%) of trichlorobenzoate **4c** were isolated and 15.1 mg (24%) of **4b** were recovered. IR (KBr): 1773, 1700, 1252, 1109 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.75–1.02 (m) therein 0.95 and 0.97 (2 s, CH_3-18, CH_3-19), 1.10–2.35 (om) therein 2.33 (t, 2H, CH_2-2^B, $^3J^B_2$=7.1 Hz), 2.69–3.02 (m, 3H, CH_2-6^A, 17α-H), 3.10–3.21 (m, 1H, CH-4^A), 4.27–4.35 (m, 1H, CH-$3a^A$), 4.47–4.57 (m, 1H, CH-$6a^A$), 4.97+5.24 (AB, 2H, CH_2-21, $^2J_{AB}$=17.8 Hz), 5.07 (bs, 1H, 3α-H), 5.48 (bs, 1H, NH), 5.77 (bs, 1H, NH), 7.46–7.57 (m, 2H, arom. H_m, ring G), 7.60–7.69 (m, 1H, arom. H_p, ring G), 7.78–7.84 (m, 2H, arom. H_o, ring G), 7.89+8.25 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=8.4 Hz). ^{13}C NMR (50.3 MHz, APT, HETCOR, $CDCl_3$): δ=15.9 (−) (C-18), 21.6 (+), 21.8 (+), 24.3 (−) (C-19), 25.4 (+), 25.5 (+), 25.7 (+), 27.4 (+), 28.8 (+), 28.9 (+), 31.0 (+), 34.9 (+) (C-2^B), 35.6 (+), 36.1 (−) and 37.3 (−) (C-5, C-9), 40.4 (+) (C-12), 41.0 (+) (C-6^A), 42.1 (+) (C-8), 47.6 (−) (C-17), 50.5 (+) (C-13), 55.9 (−) (C-4^A), 60.6 (−) (C-$6a^A$), 62.5 (−) (C-$3a^A$), 70.4 (+) (C-21), 70.7 (−) (C-3), 85.7 (+) (C-14), 129.0 (−) (C-3^G), 130.4 (−) and 130.8 (−) (C-2^F, C-3^F), 130.9 (−) (C-2^G), 131.5 (+) and 137.2 (+) and 142.9 (+) (C-1^F, C-4^F, C-1^G), 133.7 (−) (C-4^G), 135.4 (+) (C-22), 155.5 (+) (C-20), 163.0 (+) (*C*OAr^F), 164.0 (+) (C-2^A), 168.0 (+) (C-23), 173.6 (+) (C-1^B), 196.3 (+) (*C*OAr^G). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=255.5 nm (12200). $C_{47}H_{56}N_2O_9S$ (825.04), FAB MS: *m/z* (%)=847 (2; $[M+Na]^+$), 825 (1; $[M+H]^+$).

22-(4-Benzoylbenzoyloxy)-14-hydroxy-3β-(2,4,6-trichlorobenzoyloxy)-5β,14β-card-20(22)-enolide (4c). ^1H NMR (200 MHz, $CDCl_3$): δ=0.93 and 0.97 (2 s, 3H each, 18-CH_3 and 19-CH_3), 1.15–2.22 (om), 2.94–3.04 (m, 1H, 17α-H), 4.97+5.23 (AB, 2H, CH_2-21, $^2J_{AB}$=17.7 Hz), 7.35 (s, 2H, arom. trichlorobenzoyl-H), 7.46–7.57 (m, 2H, arom. H_m, ring G), 7.59–7.69 (m, 1H, arom. H_p, ring G), 7.78–7.85 (m, 2H, arom. H_o, ring G), 7.89+8.25 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=8.4 Hz). $C_{44}H_{43}O_8Cl_3$ (806.18).

14-Hydroxy-3β-{6-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido]hexanoyloxy}-5β,14β-card-20(22)-enolide (6a). To a suspension of *N*-biotinylaminocapronic acid (50.0 mg; 140 μmol) in THF (5 ml) triethylamine (21.2 mg; 210 μmol) and 2,4,6-trichlorobenzoyl chloride (34.2 mg; 140 μmol) were added dropwise and the mixture was stirred at 20°C for 4 h. After evaporation the residue was redissolved in toluene (5 ml) and a solution of digitoxigenin (37.5 mg; 100 μmol) and 4-(1-pyrrolidinyl)pyridine (29.6 mg; 200 μmol) in toluene (2 ml) was added. The mixture was stirred at 20°C for 20 h. Usual work-up and LC (ethyl acetate–methanol 5:1) provided **6a** (12.2 mg, 17%). IR (KBr): 1731, 1684, 1632, 1457, 1375 cm^{-1}. ^1H NMR (200 MHz, HH COSY, $CDCl_3$): δ=0.87 (s, 3H, CH_3-18), 0.96 (s, 3H, CH_3-19), 1.15–2.38 (om) therein 2.32 (t, CH_2-2^C, $^3J^C_{2,3}$=7.3 Hz), 2.72–3.00 (m, 3H, CH_2-6^A, 17α-H), 3.10–3.30 (m, 3H, CH-4^A, CH_2-2^B), 4.30–4.38 (m, 1H, CH-$3a^A$), 4.50–4.59 (m, 1H, CH-$6a^A$), 4.81+5.00 (AB (ABX), 2H, CH_2-21, $^2J_{AB}$=17.8 Hz, $^4J_{AX}$=1.5 Hz), 5.09 (broad s, 3α-H), 5.88 (broad s, 1H, 22-H). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=16.2 (−) (C-18), 21.5 (+), 21.7 (+), 24.2 (−) (C-19), 25.0 (+), 25.5 (+), 26.0 (+), 26.8 (+), 27.3 (+), 28.4 (+), 29.6 (+), 30.9 (+), 33.5 (+), 35.0 (+), 35.6 (+), 36.0 (−) and 37.3 (−) (C-5, C-9), 36.2 (+), 39.8 (+), 40.4 (+) (C-12), 40.9 (+) (C-6^A), 42.2 (−) (C-8), 50.0 (−) (C-17), 51.3 (+) (C-13), 55.8 (−) (C-4^A), 60.8 (−) (C-$6a^A$), 62.3 (−) (C-$3a^A$), 70.7 (−) (C-3), 73.9 (+) (C-21), 85.9 (+) (C-14), 118.2 (−) (C-22), 164.0 (+) (C-2^A), 173.8 (+), 175.1 (+) (C-20). $C_{47}H_{56}N_2O_9S$ (825.04).

22-(4-Benzoylbenzoyloxy)-14-hydroxy-3β-{6-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido]hexanoyloxy}-5β,14β-card-20(22)-enolide (6b). 4b (60 mg; 100 μmol) was converted into **6b** as described for **6a**. LC (petroleum ether–ethyl acetate–methanol 1:1:0→0:5:1) furnished **6b** (14 mg, 14%). Furthermore, 26 mg (32%) of the trichlorobenzoate **4c** were isolated. IR (KBr): 1769, 1698, 1452, 1253, 1113, 1017, 709 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ=0.82–1.00 (m) therein 0.96 and 0.97 (2 s, CH_3-18, CH_3-19), 1.20–2.22 (om), 2.32 (t, CH_2-2^C, $^3J_{2C,3^C}$=7.3 Hz), 2.64–3.01 (m, 3H) therein 2.73+2.91 (AB (ABX), CH_2-6^A, $^2J_{AB}$=13.0 Hz, $^3J_{BX}$=4.9 Hz) and (m, 17α-H), 3.15–3.20 (m, 1H, CH-4^A), 4.27–4.35 (m, 1H, CH-$3a^A$),

4.47–4.56 (m, 1H, CH-6a^A), 4.96+5.23 (AB, 2H, CH_2-21, $^2J_{AB}$=17.7 Hz), 5.07 (broad s, 3α-H), 5.13 (broad s, 1H, NH, exchanges with D_2O), 5.40 (broad s, 1H, NH, exchanges with D_2O), 7.44–7.57 (m, 2H, arom. H_m, ring G), 7.60–7.69 (m, 1H, arom. H_p, ring G), 7.78–7.84 (m, 2H, arom. H_o, ring G), 7.88+8.25 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=8.4 Hz). ^{13}C NMR (50.3 MHz, APT, $CDCl_3$): δ=15.7 (−) (C-18), 21.5 (+), 21.7 (+), 24.1 (−) (C-19), 25.3 (+), 25.4 (+), 25.6 (+), 26.7 (+), 28.7 (+), 28.8 (+), 30.1 (+), 30.9 (+), 33.6 (+), 34.7 (+), 35.6 (+), 36.0 (−) and 37.3 (−) (C-5, C-9), 40.2 (+) and 40.9 (+) (C-12, C-6^A), 42.0 (−) (C-8), 47.6 (−) (C-17), 50.4 (+) (C-13), 55.7 (−) (C-4^A), 60.5 (−) (C-6a^A), 62.4 (−) (C-3a^A), 70.3 (+) (C-21), 70.7 (−) (C-3), 85.7 (+) (C-14), 129.1 (−) (C-3^G), 130.4 (−) and 130.6 (−) (C-2^F, C-3^F), 130.9 (−) (C-2^G), 131.5 (+) and 137.2 (+) and 143.0 (+) (C-1^F, C-4^F, C-1^G), 133.7 (−) (C-4^G), 135.5 (+) (C-22), 155.4 (+) (C-20), 163.1 (+) and 163.8 (+) (C-2^A, *C*OAr^F), 168.0 (+) (C-23), 173.7 (+) (C-1^C), 196.4 (+) (*C*OAr^G). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=254 nm (11700). $C_{53}H_{67}N_3O_{10}S$ (938.20).

14-Hydroxy-3β-(4-isothiocyanatobenzoyloxy)-5β,14β-card-20(22)-enolide (7a). A solution of digitoxigenin (37.5 mg, 100 μmol) and 4-dimethylaminopyridine (12.2 mg, 100 μmol) in pyridine (2 ml) at 0°C to a solution of 4-isothiocyanatobenzoyl chloride (21.7 mg, 110 μmol) in pyridine (2 ml) was added dropwise. A yellow colour developed. The mixture was stirred at 20°C for 3 h. Usual workup and LC (petroleum ether–ethyl acetate 2:1→1:2) yielded **7a** (36.3 mg, 68%). IR (KBr): 2100, 1716, 1601, 1278, 1119 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ=0.88 (s, 3H, CH_3-18), 1.01 (s, 3H, CH_3-19), 1.13–2.27 (om), 2.75–2.85 (m, 1H, 17α-H), 4.82+5.00 (AB (ABX), 2H, CH_2-21, $^2J_{AB}$=18.2 Hz, $^4J_{AX}$=1.4 Hz), 5.34 (broad s, 1H, 3α-H), 5.88 (s, 1H, 22-H), 7.27–8.02 (AA′BB′, 4H, arom. H^D, $^3J_{AB}$=8.4 Hz, $^4J_{AA'}$=2.1 Hz). ^{13}C NMR (50.3 MHz, $CDCl_3$): δ=16.2 (C-18), 21.7, 21.8, 24.4 (C-19), 25.7, 26.9, 27.4, 31.1, 31.3, 33.6, 35.8, 36.2 and 37.7 (C-5, C-9), 40.4 (C-12), 42.3 (C-8), 50.1 and 51.4 (C-13, C-17), 72.0 (C-3), 73.9 (C-21), 85.9 (C-14), 118.2 (C-22), 126.1 (C-3^D), 130.0, 131.4 (C-2^D), 136.0 (SCN), 165.1 (CO^D), 174.9 and 175.0 (C-20, C-23). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=229 nm (23400), 283 nm (16800), 294 nm (17300). $C_{31}H_{37}NO_5S$ (535.70), FAB MS: *m/z* (%): 558 (12; $[M+Na]^+$), 536 (16; $[M+H]^+$). HRMS: calcd for $C_{31}H_{38}NO_5S$ ($[M+H]^+$) 536.2471, found 536.2482.

14-Hydroxy-3β-[4-(3-{8-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido]-3,6-dioxaoctyl}thioureido)benzoyloxy]-5β,14β-card-20(22)-enolide (8a). A solution of **7a** (21.5 mg, 40 μmol) in DMF (3 ml) was added dropwise within 10 min to a suspension of *N*-(+)-biotinyl-3,6-dioxaoctane-1,8-diamine (EZ-Link™ Biotin PEO-Amin, PIERCE, 16.5 mg, 44 μmol) in DMF (2 ml). The colour of the reaction mixture turned to intensely yellow. After another 10 min at 20°C the mixture became colourless and clear. Solvent evaporation and LC (ethyl acetate–isopropanol–methanol 3:6:1) provided **8a** (26.8 mg, 74%). IR (KBr): 1683, 1645, 1276, 694 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ=0.88 (s, 3H, CH_3-18), 1.00 (s, 3H, CH_3-19), 1.17–2.40 (om), 2.60–2.55 (m, 3H, 17α-H, CH_2-6^A), 3.03–3.17 (m, 1H, 4^A-H), 3.30–3.92 (om, 2× N–CH_2, 4× O–CH_2), 4.22–4.33 (m, 1H, 3a^A-H), 4.43–4.56 (m, 1H, 6a^A-H), 4.82+5.00 (AB, 2H, CH_2-21, $^2J_{AB}$=18.3 Hz), 5.31 (broad s, 1H, 3α-H), 5.62 (broad s, 1H, NH^A), 5.87 (s, 1H, 22-H), 6.54 (broad s, NH^A), 6.78 (broad s, NH^C), 7.38 (broad s, NH^C), 7.65+7.99 (AA′BB′, 4H, arom. H^D, $^3J_{AB}$=8.6 Hz), 9.63 (s, NH^D). ^{13}C NMR (50.3 MHz, APT, $(CD_3)_2SO$): Many of the signals were broad. The following signals could clearly be identified: δ=16.4 (−) (C-18), 21.5 (+), 21.6 (+), 24.3 (−) (C-19), 25.9 (+), 26.9 (+), 28.6 (+), 28.8 (+), 35.4 (−), 35.6 (+), 35.7 (+), 44.2 (+), 50.1 (+) (C-13), 50.9 (−) (C-17), 56.1 (−) (C-4^A), 59.8 (−) (C-6a^A), 61.7 (−) (C-3a^A), 69.0 (+), 69.8 (+), 70.2 (+), 71.2 (−) (C-3), 73.8 (+) (C-21), 84.4 (+) (C-14), 116.9 (−) (C-22), 130.4 (−), 144.2†, 163.3 (+), 165.3†, 172.8 (+), 174.5†, 177.1 (+). UV (CH_3CN): $\lambda_{max.}$ ($\epsilon_{max.}$)=262.5 nm (qual.)‡, 293.5 nm (qual.)‡. $C_{47}H_{67}N_5O_9S_2$ (910.21), ESI HRMS: calcd for $C_{47}H_{67}N_5O_9S_2Na$ ($[M+Na]^+$) 932.4248, found 932.4276, calcd for $C_{47}H_{68}N_5O_9S_2$ ($[M+H]^+$) 910.4459, found 910.4436.

22-(4-Benzoylbenzoyloxy)-14-hydroxy-3β-(4-isothiocyanatobenzoyloxy)-5β,14β-card-20(22)-enolide (7b). **4b** (73 mg, 122 μmol) was converted into **7b** as described for **7a**. Since after 27 h the reaction was incomplete (TLC) another crop of the acid chloride (10 mg, 50 μmol) was added and stirring was continued at 20°C for further 20 h. Usual work-up and LC (petroleum ether–ethyl acetate 2:1→1:2) provided 63.6 mg (68%) of the very sensitive **7b**, that could only be characterized by ^{1}H NMR (200 MHz, $(CD_3)_2SO$), characteristic signals: δ=0.88 and 0.93 (2 s, 6H, CH_3-18, CH_3-19), 1.05–2.27 (om), 2.85–3.00 (m, 1H, 17α-H), 5.19+5.24 (AB, 2H, CH_2-21, $^2J_{AB}$=18.3 Hz), 7.47–7.64 (m, arom. H), 7.67–7.84 (m, arom. H), 7.89–8.05 (m, arom. H), 7.23–7.31 (BB′ (AA′BB′), arom. H). $C_{45}H_{45}NO_8S$ (759.92).

22-(4-Benzoylbenzoyloxy)-14-hydroxy-3β-[4-(3-{8-[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido]-3,6-dioxaoctyl}thioureido)benzoyloxy]-5β,14β-card-20(22)-enolide (8b). Isothiocyanate **7b** (35.2 mg, 46 μmol) was converted into **8b** as described for **8a**. Solvent evaporation and LC (ethyl acetate–isopropanol 1:1→1:2) furnished 23.2 mg (44%) of **8b**. IR (KBr): 1762, 1694, 1661, 1539, 1450, 1270, 1114, 1016 cm^{-1}. ^{1}H NMR (300 MHz, $(CD_3)_2SO$, 80°C): many signals were broad, characteristic signals: δ=0.91 (s, 3H, CH_3-18), 0.97 (s, 3H, CH_3-19), 1.05–1.95 (om), 4.01 (broad s, 1H, NH), 4.12–4.18 (m, 1H, CH-3a^A), 4.28–4.36 (m, 1H, CH-6a^A), 5.07–5.28 (m, 3H, CH_2-21, 3α-H), 6.14 (broad s, 2H, 2 NH), 7.49–7.64 (m, arom. H), 7.69–7.98 (om, arom. H), 7.92+8.24 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=5.6 Hz), 9.90 (broad s, NH), ^{1}H NMR (400 MHz, $CDCl_3$) characteristic signals: δ=0.97 and 0.98 (2 s, CH_3-18, CH_3-19), 0.80–2.35 (om), 2.65–3.15 (om), 3.28–3.93 (om), 4.28 (broad s, 1H, CH-3a^A), 4.48 (broad s, 1H, CH-6a^A), 4.97+5.25 (AB, 2H, CH_2-21, $^2J_{AB}$=8.9 Hz), 5.29 (broad s, 3α-H), 7.38 (broad s, 2H, 2x NH), 7.48–8.05 (om, arom. H) therein 7.80 (BB′ (AA′BB′), arom. H^D), 7.88+8.24 (AA′BB′, 4H, arom. H, ring F, $^3J_{AB}$=4.1 Hz), 8.65 (broad s, NH), 9.72 (broad s, NH). UV (CH_3CN): $\lambda_{max.}$

† Observable only in ^{13}C, not in APT.
‡ Qualitative (due to low solubility).

($\epsilon_{max.}$)=258 nm (qual.)[§], 302 (qual.)[§]. $C_{61}H_{75}N_5O_{12}S_2$ (1134.43), ESI HRMS: calcd for $C_{61}H_{76}N_5O_{12}S_2$ ($[M+H]^+$) 1134.4932, found 1134.4894.

Acknowledgements

We wish to thank Dr Sabine Giesa for the ESI mass spectra. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

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[§] Qualitative (due to low solubility).