Transformation of Aminosteroids into Pharmacologically Active Amides of Phenolic Acids

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5α-cholestan-3β-yl-amine, 5α-cholestan-3α-yl-amine, 3α- and 3β-amino-(2'-aminoethyl)-cholest-5-en, Amides of Cinnamic Acid Derivatives, Antibacterial Activity

Amides of cinnamic acid derivatives with 3α- and 3β-cholestanylamines, as well as with 3α- and 3β-amino-(2'-aminoethyl)-cholest-5-en were synthesized using dicyclohexylcarbodiimide (DCC) and 1-hydroxy-benzotriazole as efficient additives. Their structure was determined by UV and ^{1}HNMR. 3β-Amino-(2'-aminoethyl)-cholest-5-en, amides of p-hydroxy-cinnamic acid **4** and **9**, and N-cholest-5-en-3α-aminoethyl-di-(3''-phenyl-trans-2''-propene)-amide **10** showed moderate antibacterial activity against *Staphylococcus aureus.*

Introduction

Studies reported on an isolation from natural sources and on synthesis of a series of aminosteroids and their derivatives showed that these substances possessed interesting biological activities: antimicrobial (Kong and Anderson, 1993), tranquillising, anticonvulsant, anesthetic (Overbeek and Bonta, 1964; Baters *et al.,* 1961; Hewett and Savage, 1968), and antiarrhythmic (Campbell *et al.,* 1979; Mokotoff *et al.,* 1990). On the other hand recently we elaborated a new method for a synthesis of steroid esters of cinnamic acid derivatives using the Wittig reaction under sonochemical conditions (Elenkov *et al.,* 1995). The established biological activities of the synthesized compounds (antioxidant (Kalichin *et al.,* 1997), antiviral (Galabov *et al.,* 1998) and antitumor (Ivanova *et al.,* 1997) urged us to look for other analogues of phenolic acids.

It was our objection to synthesize 3-aminosterols and to modify the amino function by coupling it to cinnamic acid derivatives with a view to study the antibacterial activity of the obtained amides.

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Experimental

^{1}H-NMR spectra were recorded on Bruker 250 MHz for solutions in $CDCl_3$ with TMS as internal standard. The UV spectra in EtOH solutions were measured with a Specord UV-VIS spectrophotometer.

Preparation of 3α- and 3β-steroid amines

Preparation of 3α- and 3β-5α-cholestanylamines

A solution of the oxime of cholest-3-one (882 mg, 2.2 mmol) in boiling methanol was treated with Na, added in small pieces. After 4 hours the solution was poured into ice water. The resulting precipitate was dissolved in ethylacetate and the solution acidified with 5% HCl. The organic layer contained 3α-cholestanylamine and traces of β-isomer. The water layer was neutralised with 5% NaOH and extracted with ethylacetate. The organic layer contained only 3β-cholestanylamine. The ratio of β:α isomers was 3:1, w/w. The total yield of 3α- and 3β-isomers was 92%.

5α-cholestan-3β-yl-amine (oil).

^{1}H-NMR ($CDCl_3$): 0.64 (3H, s, CH_3-18), 0.79 (3H, s, CH_3-19), 0.81 (6H, d, J=7.8 Hz, CH_3-26, 27), 0.88 (3H, d, J=8 Hz, CH_3-21), 2.6 (2H, m, NH_2), 3.0 (1H, m, 3α-H).

5α-cholestan-3α-yl-amine (oil).

^{1}H-NMR ($CDCl_3$): 0.64 (3H, s, CH_3-18), 0.78 (3H, s, CH_3-19), 0.86 (6H, d, J=7.8 Hz, CH_3-26, 27), 0.88 (3H, d, J=8 Hz, CH_3-21), 2.9 (1H, m, 3β-H).

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Preparation of 3α- and 3β-amino-(2'-aminoethyl)-cholest-5-en

Reaction was carried out with 4 mmol cholesteryl-p-toluenesulfonate and 25–30 molar equivalent of ethylenediamine in refluxing dioxane for 2h under N_2 with stirring. 3α- and 3β-amino-(2'-aminoethyl)-cholest-5-en (compounds **5** and **6**) were isolated by cc on Al_2O_3.

General procedure for preparation of amides of cinnamic acid derivatives with steroid amines

Phenolic acid (0.20 mmol), 0.16 mmol 1-hydroxy-benzotriazole (0.26 mmol) and dicyclohexylcarbodiimide (DCC, 0.18 mmol) were dissolved in dry THF under stirring until a white precipitate was obtained. To the mixture amine (0.13 mmol) in THF was added under argon and stirring at room temperature. After 3h the precipitate was filtered off and washed with CH_2Cl_2. The combined solutions were washed with $NaHCO_3$, dried over Na_2SO_4, evaporated to dryness and the residue subjected to cc (silica). All products were characterized by their UV- and ^{1}H-NMR spectra.

N-5α-cholestan-3α-yl-3''-phenyl-*trans*-2''-propene-amide (**1**)

UV (MeOH), λ_{max}: 217, 223.5, 227 nm.
^{1}H-NMR ($CDCl_3$): δ 0.65 (3H, s, CH_3-18), 0.82 (3H, s, CH_3-19), 0.86 (6H, d, *J*=6.6 Hz, CH_3-26, 27), 0.90 (3H, d, *J*=6.5 Hz, CH_3-21), 4.28 (1H, bs, 3β-H), 5.88 (1H, d, N**H**-CO), 6.42 (1H, d, *J*=16.0 Hz, H-2''), 7.47–7.53 (4H, arom.), 7.61 (1H, d, *J*= 16.0 Hz, H-3'').

N-5α-Cholestan-3β-yl-3''-phenyl-*trans*-2''-propene-amide (**2**)

UV (MeOH), λ_{max}: 217, 223, 227 nm.
^{1}H-NMR ($CDCl_3$): δ 0.58 (3H, s, CH_3-18), 0.73 (3H, s, CH_3-19), 0.79 (6H, d, *J*=6.6 Hz, CH_3-26, 27), 0.83 (3H, d, *J*=6.5 Hz, CH_3-21), 3.82 (1H, bs, 3α-H), 5.34 (1H, d, N**H**-CO), 6.20 (1H, d, *J*=16.0 Hz, H-2''), 7.19- 7.31 (2H, m, arom.), 7.40- 7.49 (3H, m, arom.), 7.52 (1H, d, *J*=16.0 Hz, H-3'').

N-5α-cholestan-3β-yl-3''-(4'''-hydroxy-3'''-methoxy)-phenyl-*trans*-2''-propene-amide (**3**)

UV (MeOH), λ_{max}: 218, 228, 294, 320 nm.
^{1}H-NMR ($CDCl_3$): δ 0.63 (3H, s, CH_3-18), 0.79 (3H, s, CH_3-19), 0.86 (6H, d, *J*=6.6 Hz, CH_3-26, 27), 0.89 (3H, d, *J*=6.5 Hz, CH_3-21), 3.89 (1H, s, OCH_3), 3.91 (1H, bs, 3α-H), 5.45 (1H, d, N**H**-CO), 5.85 (1H, s, OH), 6.21 (1H, d, *J*=16.0 Hz, H-2''), 6.87–6.94 (3H, m, arom.), 7.51 (1H, d, *J*=16.0 Hz, H-3'').

N-5α-cholestan-3β-yl-3''-(4'''-hydroxy)-phenyl-*trans*-2''-propene-amide (**4**)

UV (MeOH), λ_{max}: 227, 292, 310 nm.
^{1}H-NMR ($CDCl_3$): δ 0.65 (3H, s, CH_3-18), 0.80 (3H, s, CH_3-19), 0.86 (6H, d, *J*=6.6 Hz, CH_3-26, 27), 0.88 (3H, d, *J*=6.5 Hz, CH_3-21), 3.74 (1H, bs, 3α-H), 5.35 (1H, d, N**H**-CO), 6.20 (1H, d, *J*=16.0 Hz, H-2''), 6.84 (2H, d, *J*=8.6 Hz, arom.), 7.37 (2H, d, *J*=8.6 Hz, arom.), 7.53 (1H, d, *J*=16.0 Hz, H-3'').

Amide of cinnamic acid with 3α-amino-(2'-aminoethyl)-cholest-5-en (**7**)

UV (MeOH), λ_{max} : 217, 223, 279 nm.
^{1}H-NMR spectra ($CDCl_3$): δ 3.85 (1H, m, 3β-H), 3.50 (2H, m, -CH_2-1'), 3.99 (1H, d, -C-(O)-NH-CH_2-CH_2-N**H**-), 4.21 (2H, m, -CH_2-2'), 5.35 (1H, m, H-6), 5.90 (1H, d, N**H**-CO), 6.40 (1H, d, *J*=15.5 Hz, H-2''), 7.34- 7.37 (2H, m, arom.), 7.51-7.59 (3H, m, arom.), 7.55 (1H, d, *J*=15.5 Hz, H-3'').

Amide of cinnamic acid with 3β-amino-(2'-aminoethyl)-cholest-5-en (**8**)

UV (MeOH), λ_{max}: 218, 224, 286 nm.
^{1}H-NMR spectra ($CDCl_3$): δ 3.48 (3H, m, 3α-H; -CH_2-1'), 4.00 (1H, d, -C-(O)-NH-CH_2-CH_2-N**H**-), 4.25 (2H, m, -CH_2-2'), 5.35 (1H, m, H-6), 5.53 (1H, d, N**H**-CO), 6.46 (1H, d, *J*=16.0 Hz, H-2''), 7.36- 7.41 (2H, m, arom.), 7.50- 7.54 (3H, m, arom.), 7.55 (1H, d, *J*=16.0 Hz, H-3'').

Amide of p-hydroxy-cinnamic acid with 3α-amino-(2'-aminoethyl)-cholest-5-en (**9**)

UV (MeOH), λ_{max}: 227, 292, 314 nm
^{1}H-NMR spectra ($CDCl_3$): δ 3.45 (2H, m, -CH_2-1'), 3.74 (1H, m, 3β-H), 4.06 (1H, d, -C-(O)-NH-CH_2-CH_2-N**H**-), 4.28 (2H, m, -CH_2-2'), 5.42 (1H, m, H-6), 5.61 (1H, d, N**H**-CO), 6.24 (1H, d, *J*=15.5 Hz, H-2''), 6.84 (2H, d, *J*=8.5 Hz, arom.), 7.40 (2H, d, *J*=8.6 Hz, arom.), 7.54 (1H, d, *J*=15.5 Hz, H-3'').

Antibacterial test

For investigation of the antibacterial activity we used an agar cup method ([a]Norris *et al.*, 1972) and a method of serial dilutions ([b]Norris *et al.*, 1972). As a test microorganisms, bacteria *Staphylococcus aureus* 209 (G+) and *Esch. coli* WF+ (G-) were used.

By the agar-cup method 25 ml of the nutrient agar were poured out in Petri-dishes with a diame-

ter 100 mm. Six cups with a diameter 10 mm were formed in which 0.1 ml solution of the investigated compound was added. The agar surface was preliminary inoculated with a suspension of the corresponding strain with concentration 10^5 CFU. After the addition of the dissolved compounds the Petri-dishes were stored for 2 h in a refrigerator for diffusion. After 24 h incubation at 37 °C the diameters of the inhibitory zone of 0.5 mg of each substance were measured.

By the method of the serial dilutions 10 test-tubes, one of them being a control, were prepared with 2 ml nutrient broth. In the first tube the concentration of the investigated compounds was 1000 μg/ml, in the second- 500 μg/ml etc. (two- fold dilutions). To every one of the 9 test-tubes a suspension of *Staphylococcus aureus* with a concentration of 10^5 CFU was added. After 24 h incubation at 37 °C the test- tubes with the minimum inhibitory concentration was determined with the naked eye.

Result and Discussion

It is known that the reduction of the oxime with alkali metals and proton donors gives predominantly the equatorial conformation (Waid and Taurins, 1960), while reduction with $LiAlH_4$ furnishes mixtures, containing both equatorial and axial amines, which are usually difficult to separate (Pinkus and Pinkus, 1962).

Since the objection of the present investigation was mainly the equatorial amine as intermediate to be synthesized, the oxime of cholestan-3-one was reduced with sodium in alcohol. Instead of the mentioned in the literature n-amyl alcohol (Waid and Taurins, 1960) (which removal is very difficult even at high temperature under reduced pressure), we used methanol for the reduction. In the TLC of the reaction mixture 2 spots were observed (Dragendorf) with R_f 0.45 (orange) and 0.50 (yellow). (The TLC sheets were kept on ammonia and then developed in CH_2Cl_2: MeOH 5:1, v/v). According to literature data (Pinkus and Pinkus, 1962) the unpolarer product corresponds to α-amine, while the polarer product- to β-amine. With the progress of the reduction we observed an increase of the α-isomer. The reaction was carried out until the starting material was consumed. As the chromatographic separation of the both isomers was difficult, we tried to develop a new method for obtaining a pure α- and β-isomers. By treating the reaction products with conc. HCl the hydrochlorides of the both amines were precipitated. By treating the EtOAc solution of reaction mixture with 5% HCl we found the obtained hydrochloride of β-amine passed into water layer and the hindered α-amine stayed unchanged in organic layer. By this way it was possible to prepare pure 5α-cholestane-3β-amine and 5α-cholestane-3α-amine. The ^{1}H-NMR spectra of the corresponding N- acetamides were identical with the cited in the literature.

For the coupling of phenolic acids with amines a method for a peptide synthesis was applied using DCC and 1-hydroxy-benzotriazole (Koenig and Geiger, 1970) as efficient additives. The results obtained were shown in Table I.

The obtained amides were isolated from the reaction mixture by cc on silica. In the UV spectra of the isolated compounds two regions of absorption were observed: 215–230 nm and 240–350 nm. The peaks at 217 and 223 nm in the first region correspond to the absorption of the amide group. The absorption in the second region is due to the cinnamoyl group. The very characteristic UV spectra could be used as a preliminary evidence for an amide existence in the cc fractions.

In the ^{1}H-NMR spectrum of N-5α-cholestan-3α-yl-3''-phenyl-*trans*-2''-propene-amide (**1**) the 3β-H was observed at 4.20, the proton of -NH group- at 5.85. The both doublets at 6.42 and 7.61 with J= 15.6 Hz were related to *trans* -CH = CH- group of cinnamoyl rest. In the ^{1}H-NMR spectrum of N-5α-cholestan-3β-yl-3''-phenyl-*trans*-2''-propene-amide (**2**) all above mentioned protons were upfield shifted. 3α-H was observed at 3.83. The doublet at 5.35 corresponds to the amide proton. Two one proton doublets at 6.27 and 7.50 with J= 15.6 Hz were characteristic for -CH = CH- trans protons of cinnamoyl rest. The same doublets were characteristic for the ^{1}H-NMR spectra of the amides of ferulic **3** and *p*-coumaric acid **4**.

In contrast to the results obtained by Patel *et al.* (1985) by treating of cholesteryl-*p*-toluenesulfonate with ethylenediamine in refluxing dioxane we obtained more than one product (TLC, CH_2Cl_2:MeOH 7:1, v/v, NH_3). Most probably the reaction proceeds with inversion (like the amonolysis of cholesteryl-*p*-toluenesulfonate with inver-

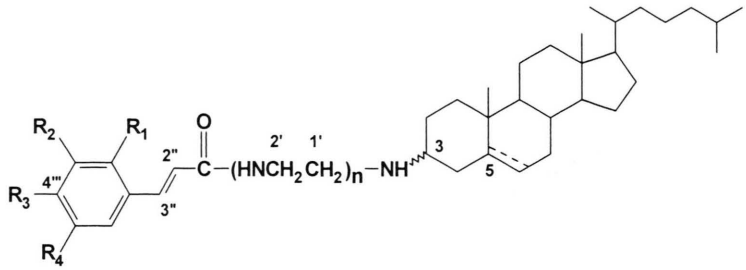


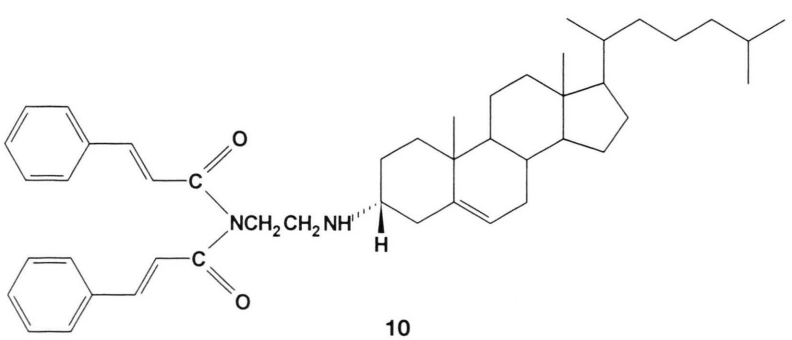
Table I. Amides of cinnamic acid derivatives with steroid amines.

Comp.			n	R_1	R_2	R_3	R_4	Yield %
1	3α	Δ^0	0	H	H	H	H	72
2	3β	Δ^0	0	H	H	H	H	97
3	3β	Δ^0	0	H	OCH_3	OH	H	73
4	3β	Δ^0	0	H	H	OH	H	35
7	3α	Δ^5	1	H	H	H	H	9
8	3β	Δ^5	1	H	H	H	H	66
9	3α	Δ^5	1	H	H	OH	H	4

sion; Haworth *et al.*, 1955) and yields mainly 3α-amino-(2'-aminoethyl)-cholest-5-en, as well 3β-amino-(2'-aminoethyl)-cholest-5-en and 3:5-cyclo-product.

From the reaction mixture 3α- and 3β-amino-(2'-aminoethyl)-cholest-5-en were isolated by cc on Al_2O_3. They were transformed to amides of cinnamic and *p*-coumaric acids (**7**, **8** and **9**) using DCC and 1-hydroxy-benzotriazole. The amides obtained were isolated using cc (silica). In the ^{1}H-NMR of α-amide of cinnamic acid (compound **8**) the amide proton was observed at 5.90, the H at C-6- at 5.35. The two doublets at 6.40 and 7.55 (J= 15.5 Hz) corresponds to the protons of trans -CH = CH-. The protons of the $-CH_2-CH_2-$ between amine and amide groups were observed as multiplettes at 3.50 and 4.21. In the ^{1}H-NMR of β-amide of cinnamic acid (compound **8**) the amide proton was up-field shifted and observed at 5.53. The doublets at 6.46 and 7.55 (J=16.0 Hz) corresponds to trans -CH = CH-.

We tried to obtained amide of cinnamic acid with 3α-amino-(2'-aminoethyl)-cholest-5-en from the corresponding cinnamoyl chloride and succeeded in carrying out the reaction in two-phase system- ethylether and 10% NaOH. After 2 h by vigorous stirring from the organic phase by cc on silica a product **10** was isolated. In its ^{1}H-NMR the amide proton was absent. Four doublets at 6.45, 6.87, 7.59 and 7.63 with J=15.5 Hz were observed and were related to the both trans -CH = CH- groups. The integral in the aromatic field showed the presence of 10 aromatic protons in the molecule. The structure of **10** was proved by COSY-^{1}H-NMR.



10

The synthesized 3α- and 3β-cholestanylamines as well as the compounds **1, 2, 3, 4** were tested for their antibacterial activity against *Staphylococcus aureus* and *Esch. coli* using the agar- cup method in triplicate. Among the investigated compounds only compound **4** demonstrated a marked activity against *Staphylococcus aureus* 209 (diameter of inhibitory zone 21±2 mm). The rest of compounds were not active. This result stimulated us to determine the minimum inhibitory concentration (MIC, μg/ml) by method of serial dilutions of compound **4**, as well of the compounds **6** [3β-amino-(2'-aminoethyl-cholest-5-en)], **9** and **10**. Among the investigated compounds **6** was found to be the most promising with MIC 62.5, followed by **10** (MIC 125.0) and **4** (MIC 250).

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