Antimicrobial Activity of Basic Cholane Derivatives Part IX

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Twenty new compounds derived from deoxycholic acid have been synthesized. They contain two basic functions: at C-24 (benzylamino, morpholino, diethanolamino, N,N-diethylethylenediamino, N-methylpiperazino) and at β-C-3 (amino, methylamino, ethylamino, benzylamino). The compounds showed interesting antimicrobial activity, as expressed in terms of the low M.I.C. values (0.9-31-μg/ml) against five *Gram*(+) and four *Gram*(-) strains, two fungi and one yeast. The compounds inhibit the production of a fluorescent pigment in *Pseudomonas aeruginosa*: this result suggests that the ability to cross the bacterium cell membrane is the first step of activity. A discussion in terms of structure-activity relationship is reported.

Antimikrobiell wirksame basische Cholanderivate

Es wurden 20 neue Verbindungen aus Deoxycholsäure synthetisiert. Sie enthalten zwei basische Funktionen: an C-24 (Benzylamino, Morpholino, Diethanolamino, N,N-Diethylethylenediamino, N-Methylpiperazino) und an β-C-3 (Amino, Methylamino, Ethylamino, Benzylamino). Alle Verbindungen weisen interessante mikrobielle Aktivität auf, ausgedrückt in den niedrigen M.I.C.-Werten (0.9-31 μg/ml) gegenüber fünf *Gram*-positiven und vier *Gram*-negativen Strängen, zwei Pilzen und einer Hefe. In einem Fall hemmten die untersuchten Verbindungen die Bildung eines fluoreszierenden Pigments bei *Pseudomonas aeruginosa*: dieses Ergebnis läßt vermuten, daß die Aktivität mit der Fähigkeit, die Zellmembran des Bakteriums zu durchdringen, beginnt. Das Verhältnis Struktur/Aktivität wird beschrieben.

A large number of new bile acid derivatives were prepared converting either the C-24 carboxyl group or C-3 hydroxy groups into a variety of amido and/or amino moieties; these new compounds showed interesting antimicrobial properties, mainly against *Gram*(+) bacterium strains.

We exhaustively examined[1,2] the role of the steroid hydroxyl pattern and the nature of the substituent at C-24 on the antimicrobial activity of common bile acid derivatives. Attention was devoted to chemical changes involving also the steroid body: the skeleton of the molecule of deoxycholic acid, that imports high activity to its derivatives, was chosen for this new series[3]. Changes were made at C-24, in the side chain, and only at the C-3 position of the steroid rings, because a regiospecific reductive amination reaction was employed[4].

Starting from the deoxycholic acid, *via* 3,12-dioxo-5β-cholan-24-N-alkylamides, we prepared in high yields twenty different 3β-amino and 3β-N-alkylamino-12-oxo-5β-24-N-alkylamides[5].

Antimicrobial activity was improved only in few cases, with respect to analogues containing the basic group in the side chain[1,2]: however, this represented an important result, because the presence of oxo or amido groups in molecules of this type cancels the activity[1,2]; furthermore a large spectrum of activity was found against fungi and yeasts, *Candida albicans*, *Penicillum luteum* and *Aspergillus orizae*. Results previously obtained are qualitatively summarized in Table 1.

Following these results we now present the preparation and activity of twenty new compounds **[I(A-D)-V(A-D)]**, namely 3β-amino- and 3β-N-alkylamino-12α-hydroxy-5β-cholan-24-N-alkylamines, carrying at C-3 the amino **(A)**, methylamino **(B)**, ethylamino **(C)**, benzylamino **(D)** groups and at C-24 the benzylamino **(I)**, morpholino **(II)**, diethanolamino **(III)**, N,N-diethylethylenediamino **(IV)** and N methylpiperazino **(V)** groups, respectively. These groups have been selected among those which proved to confer the highest activity in previous tests[1,2].

Experimental Part

Methods: M.ps.: Tottoli apparatus, uncorrected.- Infrared spectra: Perkin Elmer 197 spectrophotometer, KBr pellets.- ^{1}H-NMR spectra: Bruker spectrometer mod. AC 200.- Temp. in °C.

The purity of products was verified by tlc prior to use. Detection: 5% $HClO_4$ and heating at 120°C for 5 min or Dragendorff reagent.

Materials: 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid) (Fluka) was used without further purification. Ammonium acetate, methylamine hydrochloride, ethylamine hydrochloride, benzylamine, morpholine, N,N-diethylethylenediamine, diethanolamine and N-methylpiperazine were commercial samples of analytical grade.

General method for the synthesis of 3,12 dioxo-5β cholan-24 N-alkylamides **(I-V)**

0.01 moles of 3,12-dioxo-5β-cholan-24-oic acid (obtained by oxidation from deoxycholic acid) and 0.01 moles of tributylamine were dissolved in 100 ml of anhydrous dioxane. 1 ml of freshly distilled ethylchlorocarbonate was added dropwise under cooling and stirring. The mixture was further stirred for 10 min; then a 30% excess of the appropriate amine (benzylamine, morpholine, diethanolamine, N,N-diethylethylenediamine, and N-methylpiperazine) was added under controlled temp.; the mixture was diluted with water and extracted by ethyl acetate/ethyl ether (1:1). The org. layer was washed with 0.1 N NaOH, then with water to neutrality and dried over Na_2SO_4.

The product was separated from the starting materials and reagents by cc and then purified by the same technique. Yields were high in all cases. Experimental and analytical data for the compounds I-V have been reported in ref. 5.

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General method for the synthesis of 3β-amino and 3β-N-alkylamino-12-oxo-5β-cholan-24-N-alkylamides **I (A-D) - V (A-D)**

0.005 moles of the 3,12-dioxo-5β-cholan-24-N-alkylamides (**I-V**) were dissolved in 80 ml of anhydrous methanol; a slight excess of the selected amine salt (ammonium acetate or methyl-, ethyl-, benzyl-amine hydrochloride) was added portionwise. A large excess of $NaBH_3CN$ was added and the mixture left at room temp. until the reaction was complete: this usually took two days.

The mixture was diluted with water and extracted by ethyl acetate/ethyl ether (1:1); separation and purification were performed by cc using appropriate elution mixtures; finally the compounds were crystallized and then converted into hydrochlorides for storage. Experimental and analytical data for each compound **I (A-D)**-**V (A-D)** have been reported in ref. 5.

General method for the synthesis of 3β-amino and 3β-N-alkylamino-12α-hydroxy-5β-cholan-24-N-alkylamines **[I(A-D)-V(A-D)]**

$LiAlH_4$ (0.015 moles) was added to a solution of 3β-amino and 3β-N-alkylamino-12-oxo-5β-cholan-24-N-alkylamides **[I(A-D)-V(A-D)]** (0.01 moles) in anhydrous tetrahydrofurane (100 ml). The mixture was refluxed under stirring for 20 h, then cooled to room temp., and treated with water until a dense suspension was obtained. The product was extracted by ethyl acetate/ethyl ether; the org. phase was dried and evaporated to dryness. Reduction afforded only materials that required further purification by column chromatography (cc). The final products were stored as di (or tri) hydrochlorides.

Some representative experimental and analytical data for **I(A-D)-V(A-D)** are here reported. In each case the disappearance of the 1650 and 1700 cm^{-1} IR bands (12 oxo and 24 amide carbonyl groups) was observed.

N-Benzyl-(3β,5β,12α)-3-amino-12-hydroxy-cholan-24-amine (**IA**)

Yield 80%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (95:5); m.p.: 162-164° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.2 ($-CH_2-$), 6.0 ($-NH_2$), 6.4 (-NH), 7.5 ($-C_6H_5$).- $C_{31}H_{50}ON_2$ calc. C 79.8 H 10.80 N 6.0 found C 79.5 H 10.70 N 6.0.

N-Benzyl-(3β,5β,12α)-3-methylamino-12-hydroxy-cholan-24-amine (**IB**)

Yield 78%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (10:1); m.p.: 144-146° ($EtOAc/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.7 ($-CH_3$), 4.2 ($-CH_2-$), 6.4 (-NH), 7.5 ($-C_6H_5$).- $C_{32}H_{52}ON_2$ calc. C 79.9 H 10.90 N 5.8 found C 80.1 H 11.12 N 5.9.

N-Benzyl-(3β,5β,12α)-3-ethylamino-12-hydroxy-cholan-24-amine (**IC**)

Yield 75%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (10:1); m.p. 134-136° ($EtOAc/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.7 ($-CH_3$), 4.1-4.2 ($-CH_2-$), 6.4 (-NH), 7.5 ($-C_6H_5$).- $C_{33}H_{54}ON_2$ calc. C 79.6 H 11.27 N 5.8 found C 79.5 H 11.50 N 5.7.

N-Benzyl-(3β,5β,12α)-3-benzylamino-12-hydroxy-cholan-24-amine (**ID**)

Yield 82%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (10:1); m.p. 168-170° ($EtOAc/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.2 ($-CH_2-$), 6.4 (-NH), 7.4 ($-C_6H_5$).- $C_{36}H_{56}ON_2$ calc. C 82.0 H 10.14 N 5.0 found C 81.7 H 10.41 N 5.2.

N-[(3β,5β,12α)-3-Amino-12-hydroxy-cholan-24-yl]-morpholine (**IIA**)

Yield 77%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH; m.p. 201-203° ($EtOAc/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.0-4.3 ($-CH_2-$), 6.0 ($-NH_2$).- $C_{25}H_{50}O_2N_2$ calc. C 75.3 H 11.28 N 6.3 found C 75.0 H 11.15 N 6.2.

N-[(3β,5β,12α)-3-Methylamino-12-hydroxy-cholan-24-yl]-morpholine (**IIB**)

Yield 80%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (95:5); m.p. 158-160° ($EtOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.7 ($-CH_3$), 4.0-4.2 ($-CH_2-$), 6.42 (-NH).- $C_{29}H_{52}O_2N_2$ calc. C 75.6 H 11.38 N 6.1 found C 75.3 H 11.18 N 6.2.

N-[(3β,5β,12α)-3-Ethylamino-12-hydroxy-cholan-24-yl]-morpholine (**IIC**)

Yield 78%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (9:1); m.p. 168-170° ($EtOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.7 ($-CH_3$), 4.0-4.2 ($-CH_2-$), 6.4 (-NH).- $C_{30}H_{54}O_2N_2$ calc. C 75.9 H 11.47 N 6.9 found C 75.6 H 11.22 N 6.9.

N-[(3β,5β,12α)-3-Benzylamino-12-hydroxy-cholan-24-yl]-morpholine (**IID**)

Yield 80%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (9:1); m.p. 182-184° ($EtOAc/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.0-4.2 ($-CH_2$), 6.37 (-NH), 7.5 ($-C_6H_5$).- $C_{35}H_{56}O_2N_2$ calc. C 78.3 H 10.52 N 5.2 found C 78.1 H 10.40 N 5.3.

N-Diethanol-(3β,5β12α)-3-amino-12-hydroxy-cholan-24-amine (**IIIA**)

Yield 75%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH; m.p. 156-168° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.0-4.2 ($-CH_2-$), 6.1 ($-NH_2$).- $C_{20}H_{52}O_3N_2$ calc. C 72.4 H 11.28 N 6.0 found C 72.1 H 11.15 N 6.2.

N-Diethanol-(3β,5β,12α)-3-methylamino-12-hydroxy-cholan-24-amine (**IIIB**)

Yield 82%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (95:5); m.p. 104-106° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.7 ($-CH_3$), 4.0-4.2 ($-CH_2-$), 6.41 (-NH).- $C_{29}H_{54}O_3N_2$ calc. C 72.8 H 11.37 N 5.9 found C 75.5 H 11.15 N 5.9.

N-Diethanol-(3β,5β,12α)-3-ethylamino-12-hydroxy-cholan-24-amine (**IIIC**)

Yield 76%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (95:5); m.p. 167-169° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.70-2.80 ($-CH_3$), 4.1-4.2 ($-CH_2-$), 6.41 (-NH).- $C_{30}H_{56}O_3N_2$ calc. C 73.1 H 11.46 N 5.7 found C 72.9 H 11.15 N 5.6.

N-Diethanol-(3β,5β,12α)-3-benzylamino-12-hydroxy-cholan-24-amine (**IIID**)

Yield 78%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (95:5); m.p. 105-107° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 4.0-4.2 ($-CH_2-$), 6.4 (-NH), 7.45 ($-C_6H_5$).- $C_{35}H_{50}O_3N_2$ calc. C 75.8 H 10.54 N 5.0 found C 75.5 H 10.30 N 5.0.

N-(β-Diethylaminoethylen)-(3β,5β,12α)-3-amino-12-hydroxy-cholan-24-amine (**IVA**)

Yield 73%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (9:1); m.p. 108-110° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.6-2.7 ($-CH_3$), 4.0-4.2 ($-CH_2-$), 6.0 ($-NH_2$).- $C_{30}H_{57}ON_3$ calc. C 75.7 H 12.08 N 8.8 found C 75.4 H 12.25 N 8.7.

N-(β-Diethylaminoethylen)-(3β,5β,12α)-3-methylamino-12-hydroxy-cholan-24-amine (**IVB**)

Yield 80%; cc separation/purification: EtOAc/MeOH (1:1) / $MeOH/NH_3$ (9:1); m.p. 163-165° ($MeOH/Et_2O$).- ^{1}H-NMR: δ (ppm) = 2.6-2.8 ($-CH_3$), 4.1-4.2 ($-CH_2-$), 6.4 (-NH).- $C_{31}H_{59}ON_3$ calc. C 76.0 H 12.14 N 8.6 found C 75.8 H 11.98 N 8.7.

N-(β-Diethylaminoethylen)-(3β,5β,12α)-3-ethylamino-12-hydroxy-cholan-24-amine (**IVC**)

Yield 78%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 163-165° (MeOH).- ^{1}H-NMR: δ (ppm) = 2.5-2.8 (-CH_3), 4.1-4.2 (-CH_2-), 6.4 (-NH).- $C_{32}H_{61}ON_3$ calc. C 76.3 H 12.20 N 8.3 found C 76.0 H 12.01 N 8.4.

N-(β-Diethylaminoethylen)-(3β,5β,12α)-3-benzylamino-12-hydroxy-cholan-24-amine (**IVD**)

Yield 75%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 163-165° (MeOH/Et_2O).- ^{1}H-NMR: δ (ppm) = 2.5-2.7 (-CH_3), 4.0-4.2 (-CH_2-), 6.4 (-NH), 7.6 (-C_6H_5).- $C_{37}H_{63}ON_3$ calc. C 78.5 H 11.22 N 7.4 found C 78.3 H 11.38 N 7.6.

N_1-[(3β,5β,12α)-3-Amino-12-hydroxy-cholan-24-yl]-N_4-methylpiperazine (**VA**)

Yield 78%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 168-170° (MeOH).- ^{1}H-NMR: δ (ppm) = 2.60 (-CH_3), 6.05 (-NH_2).- $C_{29}H_{53}ON_3$ calc. C 75.8 H 11.62 N 9.1 found C 75.4 H 11.38 N 9.2.

N_1-[(3β,5β,12α)-3-Methylamino-12-hydroxy-cholan-24-yl]-N_4-methyl-piperazine (**VB**)

Yield 72%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 168-170° (MeOH).- ^{1}H-NMR: δ (ppm) = 2.5-2.7 (-CH_3), 4.0-4.2 (-CH_2-), 6.35 (-NH).- $C_{30}H_{55}ON_3$ calc. C 76.0 H 11.70 N 8.9 found C 75.9 H 11.38 N 9.0.

N_1-[(3β,5β,12α)-3-Ethylamino-12-hydroxy-cholan-24-yl]-N_4-methyl-piperazine (**VC**)

Yield 75%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 168-170° (MeOH).- ^{1}H-NMR: δ (ppm) = 2.5-2.7 (-CH_3), 4.0-4.2 (-CH_2-), 6.35 (-NH).- $C_{31}H_{57}ON_3$ calc. C 76.3 H 11.78 N 8.6 found C 76.1 H 11.51 N 8.8.

N_1-[(3β,5β,12α)-3-Benzylamino-12-hydroxy-cholan-24-yl]-N_4-methyl-piperazine (**VD**)

Yield 80%; cc separation/purification: EtOAc/MeOH (1:1) / MeOH/NH_3 (9:1); m.p. 168-170° (MeOH/Et_2O).- ^{1}H-NMR: δ (ppm) = 2.7 (-CH_3), 4.0-4.2 (-CH_2-), 6.30 (-NH), 7.5 (-C_6H_5).- $C_{36}H_{59}ON_3$ calc. C 78.6 H 10.82 N 7.6 found C 78.4 H 10.60 N 7.8.

Microbiological Tests

Gram(+) strains: *Staphylococcus aureus*, CCM 2022; *Staphylococcus aureus* "Heatley Oxford" 3R 7089, CCM 2107; *Streptococcus faecium*, CCM 1875; *Bacillus subtilis*, CCM 1999;

Gram(-) strains: *Escherichia coli*, CCM 5172; *Serratia marcescens*, CCM 303; *Proteus inconstans*, CCM 5651; *Salmonella enteritidis*, CCM 5439; *Pseudomonas aeruginosa*, CCM 1960;

Fungi: *Penicillum luteum*, ATCC 10125; *Aspergillus oryzae*, ATCC 1011;

Yeast: *Candida albicans*, ATCC 752.

Tests were performed on *Mueller-Hinton* medium by broth dilution technique[6-8]: an interval ranging from 500 to 0.030 μg/mL was chosen. A 24 h broth culture was used as inoculum containing 10^7 cells/mL. After 24 h incubation at 37 ± 0.2°, results were recorded as lowest concentration of the compound (as hydrochloride) able to inhibit growth of bacteria (minimum inhibitory concentration, M.I.C.) (Table 2). For fungi and yeast a slightly different technique was used: ref.[5].

Results and Discussion

The chemical reactions involved in the preparation of **I(A-D)-V(A-D)** have been reported[1,3]. The reductive amination used is stereospecific[4] and regioselective and involves only the C-3 oxo group: only beta epimers were obtained, according to lit. suggestions. α-Epimers could be obtained following different synthetic steps[3]: the reaction mechanism has been not yet elucidated.

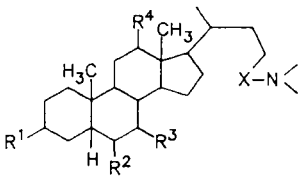

Table 1: Relationship between structure and antimicrobial activity of basic cholane derivatives and analogs.

	R^1	R^2	R^3	R^4	X	Activity
lithocholyl	αOH	H	H	H	CH_2	very active
hyodeoxycholyl	αOH	αOH	H	H	CH_2	poorly active
ursodeoxycholyl	αOH	H	βOH	H	CH_2	inactive
chenodeoxycholyl	αOH	H	αOH	H	CH_2	very active
deoxycholyl	αOH	H	H	αOH	CH_2	very active
cholyl	αOH	H	αOH	αOH	CH_2	poorly active
ursocholyl	αOH	H	βOH	αOH	CH_2	inactive
	αOH	H	H	αOH	CO	inactive (ref.1)
	=O	H	H	=O	CO	inactive (ref.3)
	βN<	H	H	=O	CO	active
	βN<	H	H	αOH	CH_2	very active [I(A-D)-V(A-D)]

For microbiological determinations, mother solutions of **I(A-D)-V(A-D)** were prepared in dimethylsulphoxide, due to their poor solubility in water: since microbiological tests were performed with the dilution method, the amount of the org. solvent in the final mixture was found not to interfere.

The antibacterial activity of basic cholane derivatives appeared to be related to the hydrophobicity of the molecules[2]. A significant role was also attributed to the basicity strength of these compounds (amines constantly appear to be more active than corresponding amides) (Table 1).

Change of the position of the basic function from C-24 to C-3 resulted in the simultaneous introduction of an amide group in C-24 and of an oxo group in C-12 **I(A-D)-V(A-D)**.

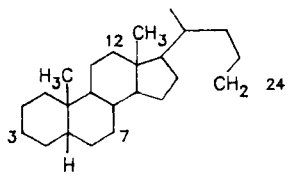

Table 2: M.I.C. values for basic cholane derivatives I(A-D)-V(A-D) and analogs

SUBSTITUENTS (*)						M.I.C. (μg/ml) (*)			
	3α	3β	7α	12α	24	S.a.	S.o.	S.f.	B.s.
IA	H	NH_2	H	OH	BzNH	3.90	3.90	3.90	3.90
IB	H	MeNH	H	OH	BzNH	7.81	7.81	7.81	3.90
IC	H	EtNH	H	OH	BzNH	3.90	3.90	7.81	1.95
ID	H	BzNH	H	OH	BzNH	1.95	1.95	1.95	0.97
	OH	H	H	OH	BzNH	62.50	15.62	7.81	7.81
	OH	H	OH	H	BzNH	250.00	125.00	125.00	15.62
	OH	H	H	H	BzNH	7.81	3.90	3.90	15.62
IIA	H	NH_2	H	OH	MORP	7.81	7.81	7.81	3.90
IIB	H	MeNH	H	OH	MORP	62.50	62.50	62.50	31.25
IIC	H	EtNH	H	OH	MORP	3.90	3.90	31.25	15.62
IID	H	BzNH	H	OH	MORP	7.81	7.81	7.81	3.90
	OH	H	H	OH	MORP	62.50	31.20	62.50	7.81
	OH	H	OH	H	MORP	15.62	15.62	15.62	3.90
	OH	H	H	H	MORP	500.00	250.00	500.00	500.00
IIIA	H	NH_2	H	OH	DEA	15.62	15.62	15.62	7.81
IIIB	H	MeNH	H	OH	DEA	15.62	15.62	15.62	15.62
IIIC	H	EtNH	H	OH	DEA	3.90	3.90	3.90	3.90
IIID	H	BzNH	H	OH	DEA	7.81	7.81	7.81	7.81
	OH	H	H	OH	DEA	62.50	62.50	62.50	62.50
	OH	H	OH	H	DEA	62.50	62.50	62.50	62.50
	OH	H	H	H	DEA	62.50	62.50	62.50	31.25
IVA	H	NH_2	H	OH	DEEDA	3.90	3.90	1.95	1.95
IVB	H	MeNH	H	OH	DEEDA	15.62	15.62	15.62	7.81
IVC	H	EtNH	H	OH	DEEDA	31.25	31.25	31.25	31.25
IVD	H	BzNH	H	OH	DEEDA	3.90	3.90	3.90	1.95
	OH	H	H	OH	DEEDA	15.62	15.62	15.62	7.81
	OH	H	OH	H	DEEDA	62.50	62.50	62.50	31.25
	OH	H	H	H	DEEDA	500.00	250.00	500.00	250.00
VA	H	NH_2	H	OH	MPIP	15.62	15.62	7.81	7.81
VB	H	MeNH	H	OH	MPIP	62.50	62.50	62.50	31.25
VC	H	EtNH	H	OH	MPIP	62.50	62.50	62.50	62.50
VD	H	BzNH	H	OH	MPIP	15.62	15.62	15.62	15.62
	OH	H	H	OH	MPIP	125.00	62.50	62.50	62.50
	OH	H	OH	H	MPIP	250.00	125.00	125.00	31.25
	OH	H	H	H	MPIP	31.25	31.25	31.25	15.62

(*) Abbreviations: M.I.C. minimal inhibitory concentration in μg/ml; S.a. *Staphylococcus aureus*; S.o. *Staphylococcus oxford*; S.f. *Streptococcus faecium*; B.s. *Bacillus subtilis*. Basic moieties: NH_2 amine; MeNH methylamine; EtNH ethylamine; BzNH benzylamine; MORP morpholine; DEA diethanolamine; DEEDA N,N diethylethylenediamine; MPIP N methylpiperazine.

The location of the basic group on one end of the steroid skeleton makes these molecules very active, also balancing the negative contributions of the C-12 and C-24 carbonyl groups[5]; again, activity was supported by bulk hydrophobic substituents, irrespective of whether they were bound to amine or amide groups.

The reduction of the two carbonyl groups in this system was afforded in order to further improve the activity in **I(A-D)-V(A-D)**: activity was found to increase with respect to the corresponding Cβ-3-amines/C-24-amido (Table 1) and hydroxy/C-24-amines. M.I.C. values for **I(A-D)-V(A-D)** and the most active hydroxy/C-24-amines are reported in Table 2.

While the primary amino group in C-3 **[IA-VA]** does not induce a change in activity (3.9-15.68 μg/ml), methylamino **[IB-VB]** and ethylamino **[IC-VC]** compounds, particularly **IB** and **IC**, showed an increased activity with respect to oxygenated compounds. Very low M.I.C. values were found when a benzylic moiety is present at C-24: particularly interesting is **ID** containing two of these moieties, which had the lowest M.I.C. values (1.95-0.97 μg/ml), depending on the bacterium strain. On the contrary the methylpiperazino residue does not appear to introduce activity.

M.I.C. values refer to the hydrochlorides. Since the active species is the unionized one, the concentration of the active species should be lower than that reported, as a function of the pKa of these compounds. By comparison with structurally related compounds, those compounds under examination, which are diamines (primary, secondary, and tertiary) - in some cases, triamines - should have a pKa ranging from 9.5 to 10.5: sufficiently high to leave only a few percents of the unionized species[9] at the pH of the microbiological tests (7.40).

Interesting results were obtained when the activity was considered against *Gram*(-) strains: **I(A-D)-V(A-D)** proved to be active as bactericides, albeit at high concentrations: 250-500 μg/ml.

Probably the most distinct feature of these compounds is the direct information concerning their mechanisms of action.

In a previous paper[2], crossing of the cell membrane was hypothesized, as the first step of antimicrobial activity, by the linear relationship existing between -log M.I.C. and log P. In fact the partition coefficient P mimics *in vitro* the *in vivo* crossing of a cell membrane: the lowest values of concentration of this class of compounds inhibiting bacterium growth are found for most hydrophobic compounds.

This was also supported the fragment constant values: the amide group imports less hydrophobicity than the corresponding $-CH_2NH-$ group[10]: this partly explains the lower activity of the compounds carrying the amide function.

Suggestions of the penetration of these compounds through the cell membrane were derived from the behaviour of some C-3β-N-alkylamino/C-24-amides: the two epimers 3β-dimethylamino- and 3α-dimethylamino-12-oxo-5β-cholan-24-amide, separated as reported[3] from the products of the amination reductive reaction, showed comparable activity, notwithstanding stereochemical differences: it was reasonable to suppose membrane crossing rather than receptor contact.

In this series, **I(A-D)-V(A-D)** were found to inhibit a pigment in *Pseudomonas aeruginosa*, at concentrations around 62.5 μg/ml. Many bacterium strains synthesize pigments[11]: in particular *Pseudomonas* produces, among other, a fluorescent pigment (pyoverdin). The wavelength of ultraviolet radiation for maximum excitation is around 400 nm, a property useful to reveal these pigments[12]. In presence of all compounds under examination, no fluorescence was observed: since the synthesis is carried out inside the cell cytoplasm[11] this can result only when the compounds have crossed the bacterium cell membrane. Furthermore, since it is well known[12] that the chemical instability of pyoverdin can be lowered by formation of a ferric complex, diamines of this series can act as chelators and compete in complex formation with pyoverdin, thus causing its degradation.

Finally the activity against the two fungi was found in the M.I.C. range 125-31.25 μg/ml; while against *Candida albicans* many compounds are active in concentrations ranging from 31.25 to 15.62 μg/ml.

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[Ph668]