Antimicrobial Activity of Basic Cholane Derivatives, VIII

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Two series of new compounds derived from deoxycholic acid have been synthesized: 3,12-dioxo-5β-cholan-24-N-substituted amides and their 3β-amino and 3β-N-alkylamino derivatives.

The first series of five compounds **1-5** carries at C-24 the residue of benzylamine, morpholine, diethanolamine, N,N-diethyl-ethylenediamine, and N-methylpiperazine.

The second series of twenty compounds **1A-D - 5A-D** was prepared by means of reductive amination starting from the compounds of the first series. This reaction proved to be regioselective and stereospecific: it attacks only C-3 of the steroid moiety and introduces the following axial β-oriented sustituents: amino, methylamino, ethylamino, and benzylamino.

The compounds of the first series showed moderate scattered antimicrobial activity; while introduction of the 3β-amino and 3β-N-alkylamino residue greatly increased activity towards *Gram* (+) strains; even yeast and fungi appear to be sensitive towards this last series of compounds.

The results have been discussed with respect to the nature of the substituents both at C-3 and C-24, the highest activity being associated to the hydrophobicity of these residues.

Antimikrobielle Aktivität basischer Cholan-Derivate, 8. Mitt.

Es wurden zwei Reihen neuer von Desoxycholsäure abstammender Verbindungen synthetisiert: 3,12-Dioxo-5β-cholan-24-N-alkylamide und 3β-Amino- und N-Alkylamino-12-oxo-5β-cholan-24-alkylamide.

Die erste Reihe von fünf Verbindungen **1-5** trägt an C-24 einen Benzylamin-, Morpholin-, Diethanolamin-, N,N-Diethylethylendiamin- bzw. N-Methylpiperazin-Rest.

Die zweite Reihe von zwanzig Verbindungen **1A-D-5A-D** wurde durch reduktive Aminierung hergestellt, ausgehend von den Verbindungen der ersten Reihe. Diese Reaktion erwies sich als regioselektiv und stereospezifisch: sie betrifft nur C-3 des Steroidanteils und führte folgende axial β-orientierte Substituenten ein: Amin, Methylamin, Ethylamin und Benzylamin.

Die Verbindungen der ersten Reihe zeigten eine befriedigende und gestreute antimikrobielle Aktivität. Die Einführung der 3β-Amino- und N-Alkylaminogruppe verstärkte die Aktivität gegenüber grampositiven Arten; offensichtlich reagieren sogar Hefen und Pilze auf diese Verbindungsreihe.

Die Ergebnisse wurden bezüglich der Art der Substituenten sowohl an C-3 als auch an C-24 betrachtet: die größte Aktivität hängt mit der Hydrophobie der Reste zusammen.

A new series of cholane derivatives has been obtained from bile acid molecules substituting the C-24 acidic carboxyl group with an amido or an amino group of different nature. These derivatives show an interesting antimicrobial activity against *Gram* (+) and, to a lesser extent, *Gram* (-) strains[1-5].

The complex role of both the side chain structure and steroidal substituent pattern has been discussed and related to the chemical-physical properties of a selected series of these compounds[6].

More recently, starting from 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid), two new classes of compounds were synthesized, carrying a β oriented basic function at C-3, namely the 3β-amino and 3β-N-alkylamino-12-oxo-5β-cholan-24-amide and the 3β-amino and 3β-N-alkylamino-12α-hydroxy-5β-cholan-24-amine[7,8].

In this paper the above series has been extended to N-substituted C-24 amides: the sequence of transformations is presented in Schema A in the case of deoxycholic acid, as an example. In steps 2 and 3 of Scheme A, almost all possible combinations have been considered, taking into account eight different bile acids and more than ten different amines[1-5].

N-substituted amides of the 3,12-dioxo-5β-cholan-24-oic acid have been prepared by means of a mixed anhydride with ethylchlorocarbonate **1-5**. A reductive amination of the oxo group at C-3 of the steroid moiety by means of $NaBH_3CN$, in presence of the appropriate amine salt, produced the compounds of the series **1A-D-5A-D**. This reaction resulted[9] to be regioselective and stereospecific. The two series of compounds are presented in Scheme B.

Experimental Part

Chemistry

Method: M.p: Tottoli apparatus, uncorrected. - IR-spectra: Perkin Elmer 197 spectrophotometer in KBr. All the compounds showed characteristic bands at 690-750; 1650; 1700 and 3300 (broad) cm^{-1}, attributed to the benzyljc moiety (when present), to amidic and keto groups, and to $-NH_2$ and -NHR groups, respectively. - ^{1}H-NMR spectra: Bruker spectrometer mod. AC 200. All the compounds showed signals at 6 or 6.4 p.p.m. of NH_2 or NH groups at C-3: signals disappeared in D_2O, CH_3, CH_2 and C_6H_5

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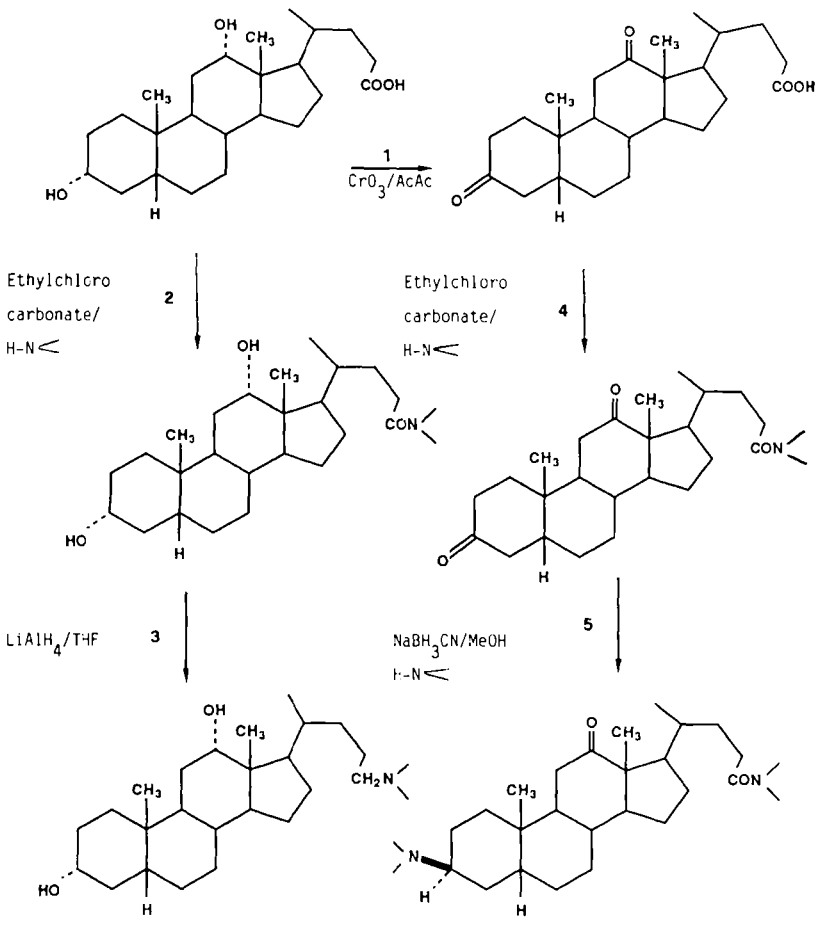
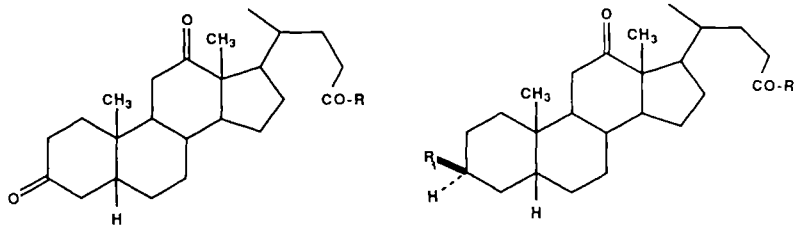
SCHEME A



SCHEME B



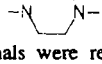
Series **1** - **5**

R = -NH-CH_2-C_6H_5 [1]


[2]

-N-[CH_2CH_2OH]$_2$ [3]

-NH-CH_2-CH_2-N[C_2H_5]$_2$ [4]


[5]

Series **1** (**A-D**)-**5** (**A-D**)

R_1 = -NH_2 [A]

= -NH-CH_3 [B]

= -NH-C_2H_5 [C]

= -NH-CH_2-C_6H_5 [D]

signals were recorded at 2.68 (doublet), 4.2 (singlet), 7.47 (multiplet) p.p.m., attributed to -NH-CH_2-CH_3 and -NH-CH_3-, to -NH-CH_2-CH_3 and -NH-CH_2-C_6H_5, and to -NH-CH_2-C_6H_5, respectively.

Elemental analysis showed C, H, and N percentage values differing less than ±0.3 from the theoretical ones.

TLC: silica gel sheets (Merck), using the appropriate developing mixtures reported in Table 1. Detection: 10% perchloric acid solution and heating for 5 min at 120°C.

Materials: 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid) was purchased form Sigma and was used without further purification. Ammonium acetate, methylamine hydrochloride, ethylamine hydrochloride, benzylamine, morpholine, N,N-diethylethylene-diamine, diethanolamine and N-methylpiperazine were commercial samples. They were crystallized or distilled under vacuum as appropriate just prior to use and stored in a refrigerator. All other reagents were of analytical grade.

Tab. 1:

	Compounds	Yield (%)	M.P	M.P (HCl)	ES	CS	EA C%	EA H% Calc. Found	EA N%
1	N-benzyl-3,12-dioxo-5β-cholan-24-amide ($C_{31}H_{43}O_3N$)	80	37-9°	-	EtOAc/EtPet	EtOAc/Et_2O	77.9	9.07	2.9
					EtOAc		77.6	8.80	2.8
1A	N-benzyl(3β, 5β) 3-amino-12-oxo-cholan-24-amide ($C_{31}H_{46}O_2N_2$)	80	30-2°	183-5°	EtOAc	EtOAc/Et_2O	77.8	9.69	5.8
					MeOH		77.5	9.80	5.9
1B	N-benzyl(3β, 5β)-3-methylamino-12-oxo-cholan-24-amide ($C_{32}H_{48}O_2N_2$)	75	30-2°	104-6°	EtOAc/MeOH (8:2); *MeOH*	MeOH/Et_2O	78.0	9.82	5.7
							77.8	9.60	5.7
1C	N-benzyl(3β, 5β)-3-ethylamino-12-oxo-cholan-24-amide ($C_{38}H_{50}O_2N_2$)	80	35-7°	162-4°	EtOAc/MeOH (8:2); *MeOH*	MeOH/Et_2O	78.2	9.95	5.5
							77.9	9.70	5.4
1D	N-benzyl(3β, 5β)-3-benzylamino-12-oxo-cholan-24-amide ($C_{38}H_{52}O_2N_2$)	80	30-1°	85-7°	EtOAc/MeOH (9:1); *MeOH*	MeOH/Et_2O	80.2	9.22	4.9
							80.1	9.48	4.8
2	N(3,12,24-trioxo-5β-cholan-24-yl) morpholine ($C_{28}H_{43}O_4N$)	85	165-7°		EtOAc/MeOH (8:2)	MeOH/H_2O	73.5	9.47	3.1
							73.2	9.60	3.0
2A	N[(3β, 5β) 3-amino-12,24-dioxo-cholan-24-yl] morpholine ($C_{28}H_{46}O_3N_2$)	80	156-8°	>230°	EtOAc/MeOH (5:5); *MeOH*	MeOH/H_2O	73.3	10.1	6.1
							73.1	10.25	6.2
2B	N[(3β, 5β) 3-methylamino-12,24-dioxo cholan-24-yl] morpholine ($C_{29}H_{48}O_3N_2$)	82	111-3°	202-4°	EtOAc/MeOH (8:2)	MeOH/H_2O	73.7	10.24	5.9
							73.4	10.35	5.9
2C	N[(3β, 5β) 3-ethylamino-12,24-dioxo-cholan-24-yl] morpholine ($C_{30}H_{50}O_3N_2$)	78	114-6°	>230°	EtOAc/MeOH (7:3); *MeOH*	MeOH/H_2O	74.0	10.36	5.8
							73.8	10.10	5.6
2D	N[(3β, 5β) 3-benzylamino-12,24-dioxo-cholan-24-yl] morpholine ($C_{35}H_{52}O_3N_2$)	76	78-80°	137-8°	EtOAc/MeOH (8:2); *MeOH*	MeOH/H_2O	76.6	9.55	5.1
							76.3	9.40	5.1
3	N-diethanol-3,12-dioxo 5β-cholan-24-amide ($C_{28}H_{45}O_5N$)	80	167-9°		EtOAc	MeOH/H_2O	70.7	9.5	42.9
					MeOH		70.5	9.7	2.7
3A	N-diethanol (3β, 5β) 3-amino-12-oxo-cholan-24-amide ($C_{28}H_{48}O_4N_2$)	75	33-5°	128-30°	EtOAc/MeOH (7:3); *MeOH*	MeOH/Et_2O	70.5	10.15	5.9
							70.7	10.32	5.7
3B	N-diethanol (3β, 5β) 3-methylamino-12-oxo-cholan-24-amide ($C_{29}H_{50}O_2N_2$)	76	33-5°	185-7°	EtOAc/MeOH (8:2); *MeOH*	MeOH/Et_2O	71.8	10.27	5.7
							70.7	10.38	5.6
3C	N-diethanol (3β, 5β) 3-ethylamino-12-oxo-cholan-24-amide ($C_{30}H_{52}O_4N_2$)	78	30-2°	>230°	EtOAc/MeOH (8:2); *MeOH*	MeOH/Et_2O	71.4	10.38	5.5
							71.1	10.50	5.6
3D	N-diethanol (3β, 5β) 3-benzylamino-12-oxo-cholan-24-amide ($C_{35}H_{54}O_4N_2$)	75	40-2°	123-5°	EtOAc/MeOH (8:2); *MeOH*	MeOH/Et_2O	74.2	9.60	4.9
							73.9	9.70	4.8
4	N(β-diethylaminoethyl) 3,12-dioxo 5β-cholan-24-amide ($C_{30}H_{50}O_3N_2$)	75	76-8°	105-7°	EtOAc/MeOH (9:1); *MeOH*	EtOAc/Et_2O	74.0	10.36	5.8
							73.8	10.48	5.7
4A	N(β-diethylaminoethyl) (3β, 5β) 3-amino-12-oxo-cholan-24-amide ($C_{30}H_{53}O_2N_3$)	85	32-3°	70-2°	MeOH	MeOH/Et_2O	73.9	10.95	8.6
							73.6	10.80	8.5
4B	N(β-diethylaminoethyl) (3β, 5β) 3-methylamino-12-oxo-cholan-24-amide ($C_{31}H_{55}O_2N_3$)	76	83-5°	128-30°	EtOAc/MeOH	MeOH/Et_2O	74.2	11.05	8.4
					(5:5) (*)		74.1	11.20	8.2
4C	N(β-diethylaminoethyl) (3β, 5β) 3-ethylamino-12-oxo-cholan-24-amide ($C_{32}H_{57}O_2N_3$)	80	80-2°	124-6°	EtOAc/MeOH	MeOH/Et_2O	74.5	11.14	8.1
					(5:5) (*)		74.3	10.59	8.2
4D	N(β-diethylaminoehtyl) (3β, 5β) 3-benzylamino-12-oxo-cholan-24-amide ($C_{37}H_{59}O_2N_3$)	75	30-1°	135-7°	EtOAc/MeOH (5:5) (*)	MeOH/Et_2O	76.9	10.29	7.3
							76.7	10.50	7.1
5	N_1(3,12,24-trioxo-5β-cholan-24-yl) N_4-methylpiperazine ($C_{29}H_{46}O_3N_2$)	85	183-5°	200-2°	EtOAc/MeOH (5:5)	MeOH/H_2O	74.0	9.85	5.9
							73.7	9.60	6.0
5A	N_1[(3β, 5β) 3-amino-12,24-dioxo-cholan-24-yl] N_4-methylpiperazine ($C_{29}H_{49}O_2N_3$)	75	120-2°	215-7°	MeOH (*)	MeOH/Et_2O	73.8	10.47	8.9
							73.6	10.30	8.8
5B	N_1[(3β, 5β) 3-methylamino-12,24-dioxo cholan-24-yl] N_4-methylpiperazine ($C_{30}H_{51}O_2N_3$)	78	32-4°	187-9°	EtOAc/MeOH (5:5) (*)	MeOH/Et_2O	74.2	10.58	8.6
							73.9	10.40	8.5
5C	N_1[(3β, 5β) 3-ethylamino-12,24-dioxo cholan-24-yl] N_4-methylpiperazine ($C_{31}H_{53}O_2N_3$)	75	38-9°	>230°	EtOAc/MeOH (5:5) (*)	MeOH/Et_2O	74.5	10.69	8.4
							74.2	10.80	8.3
5D	N_1[(3β, 5β) 3-benzylamino-12,24-dioxo cholan-24-yl] N_4-methylpiperazine ($C_{36}H_{55}O_2N_3$)	80	33-5°	160-2°	EtOAc/MeOH (7:3) (*)	MeOH/Et_2O	76.9	9.87	7.5
							76.7	9.60	7.4

abbreviations: mp: melting point of free base; mp(HCl): melting point of hydrochloride;
ES: elution solvent for separation; *ES*: elution solvent for purification; EA: elemental analysis
CS: crystallization solvent; EtOAc: ethyl acetate; EtPet: petroleum ether; Et_2O: ethyl ether; MeOH: methyl alcohol.
(*) *methyl alcohol/ concentrated ammonia solution (9:1).*

Synthesis

3,12-dioxo-5β-cholan-24-amides **1-5**

To 0.01 moles of 3,12-dioxo-5β-cholan-24-oic and 0.01 moles of tributylamine in 100 m of anhydrous dioxane, 0.01 moles of freshly distilled ethylchlorocarbonate were added dropwise under cooling and stirring. The mixture was further stirred for 10 min then a 30% excess (0.013 moles) of the appropriate amines was added at 10°C; after 3 h at room temp. the mixture was diluted with water and extracted by ethyl acetate/ethyl ether (1:1).

The final products were separated from the starting materials and reagents by column chormatography (cc) and then purified by the same technique: the appropriate mixtures are reported in Table 1. Yields were high in all cases. Table 1 also reports the free base and hydrochloride melting points as well as the solvents for crystallization.

3β-amino and 3β-N-alkylamino-12-oxo-5β-cholan-24 substituted amides **1A-D-5A-D**

0.005 moles of the 3,12-dioxo-β-cholan-24-substituted amides **1-5** were dissolved in 80 ml of anhydrous methanol with gentle warming; then a slight excess (0.006-0.007 moles) of the selected amine salt (ammonium acetate or methyl-, ethyl-, benzyl-amine hydrochloride) was added portionswise as appropriate. When necessary, the solution was buffered at pH = 7.0 with dilute methanolic KOH.

0.02 moles of $NaBH_3CN$ were added and the mixture left at room temp. until the reaction was complete: this usually took two days. A small amount of the hydride was added every 24 h.

Separation and purification were performed by cc using appropriate elution mixtures; finally the compounds were crystallized and then converted into hydrochlorides for storage (Table 1).

Microbiology

Materials: - Four *Gram* (+) strains (*Staphylococcus aureus*, CCM 2022; *Staphylococcus aureus* "Heatley Oxford" strain 3R 7089, CCM 2107; *Streptococcus faecium*, CCM 1875; *Bacillus subtilis*, CCM 1999 - vegetative form), five *Gram* (-) strains (*Escherichia coli*, CCM 5172; *Serratia marcescens*, CCM 303; *Proteus inconstans*, CCM 5651; *Salmonella enteritidis*, CCM 5439; *Pseudomonas aeruginosa*, CCM 1960), one yeast (*Candida albicans*, ATCC 752), and two fungi (*Penicillum luteum*, ATCC 10125; *Aspergillus oryzae*, ATCC 1011) were selected for *in vitro* tests.

Type cultures come from the Czechslovak Collection of Microorganisms (CCM), Brno, and from the American Type Culture Collection (ATCC), Rochville (Maryland)

Method: Tests were performed by broth dilution technique[10-12], in an interval ranging from 500 to 0.030 μg of active ingredient per one ml of diluent.

Mueller-Hinton medium was chosen for bacterial growth, and *Sabouraud* Dextrose Broth for yeast and fungi.

A 24-hour broth culture was used as inoculum for bacteria and yeast, which contained 10^7 cells/ml[13]; for fungi, the AOAC method was applied, the inoculum containing $5 \cdot 10^6$ conidia/ml.

The incubation period was 24 h at 37°C for bacteria and yeast and one week at 25°C for fungi.

Results were recorded as μg/ml, and the lowest concentration of the substances to inhibit growth was designated the "minimum inhibitory concentration" (M.I.C.)

Interpretative standards were obtained from *Buttiaux* et al.[14] and from *Cobb* et al.[15].

Results and Discussion

Chemistry: Following the observations that conjugation of a bile acid with a basic aminoacid (e.g. lysine)[16-18] introduced an antimicrobial activity, a systematic structure-activity study was started in order to optimize this property in molecular systems derived from bile acids. One or more basic functions have been introduced first[1-5] replacing the C-24 carboxyl group, then[9b] the C-3 hydroxyl group through a series of modifications.

Here two different new series of deoxycholic acid derivatives have been synthesized.

The first series has two keto groups at C-3 and C-12 of the steroidal moiety and an amide function at C-24 of the side chain: therefore it is neutral from the acid-base equilibrium point of view.

An amide function was introduced into 3,12-dioxo-5β-cholan-24-oic acid *via* the mixed anhydride method[1-5]. Amines, which showed to confer the most effective antimicrobial activity in the series of amido and amino derivatives of common bile acid reported, were selected. The method, widely used for the synthesis of conjugated bile acids, gave high yields of the five compounds **1-5**.

The second series of compounds has been prepared from **1-5** reductive amination using ammonia (A), methylamine (B), ethylamine (C), and benzylamine (D) in presence of $NaBH_3CN$, thus obtaining **1A-D-5A-D** with all the possible combinations. Dialkylamines have not been used, since they confer a very low antimicrobial activity on the molecule[10]. Compounds in this series have therefore *one* basic center in the steroidal moiety. Compounds **4A-D** and **5A-D** obtain *two* basic centers.

As reported[9], the reduction involves only the C-3 carbonyl group, while the C-12 one proved rather insensitive towards this reaction. The IR spectra of **1A-D-5A-D** show both absorptions at 1700 and 1650 cm^{-1} attributed to the C-12 keto and the C-24 amide carbonyl groups, respectively. Their intensity ratio appeared reduced with respect to that observed in **1-5**, containing two carbonyl groups on the steroid rings.

The reductive amination proved to be regioselective and stereospecific. Regioselectivity was found with respect to C-3: attempts to carry out the reductive amination on isolated C-7 or C-12 keto groups were unsuccesfull. Stereospecificity with respect to 3β configuration was demonstrated by alternative synthesis. It is well documented[9] that the reduction of 3-oxime-5β-cholan-24-oic acid with Na^0 and alcohols affords 3α-NH_2 predominatly over 3β-NH_2 epimer (9:1), while reductive amination route offered a different ratio (2:98) of the two epimers. This last method was preferred on account of its regioselectivity: the products are therefore axial 3β amino or 3β alkylamino derivatives.

Microbiology: Dioxocholanylamides appeared inactive towards bacteria, yeast, and fungi irrespective of the nature of the moiety at C-24 (**1-5**).

Antimicrobial activity was present in the series **1A-D-5A-D**, where an amine group was introduced at C-3: activity was rather articulated, depending on both the nature of the amine moiety and the strain examined. The primary amino group (-NH_2) [**1A-5A**] proved highly active towards *Gram* (+) strains, the activity being very high in each case

following this rank order: **4A** (3.9 μg/ml); **1A, IIA, 5A** (7.8 μg/ml); **2A** (15.6 μg/ml).

When a benzylamine moiety was present at C-3, activity was also high ranging from 31.2 μg/ml to 15.6 μg/ml for the series **2D-5D**; while **1D**, i.e. the compound containing the benzylamino [C-3] and benzylamido [C-24] groups appeared to be even more active (7.8 μg/ml).

The series **1B-5B** and **1C-5C** was less active than the two previous series. As far as the *Gram* (-) strains are concerned, the compounds showed only scattered activity up to 250 or 125 μg/ml.

In contrast, yeast and fungi were more sensitive towards these compounds, albeit at higher M.I.C. values found in the case of Gram (+) bacterium strains.

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