STREPTOMYCYLAMINES: DIFFERENCE IN ACTIVITY AND MODE OF ACTION BETWEEN SHORT-CHAIN AND LONG-CHAIN DERIVATIVES

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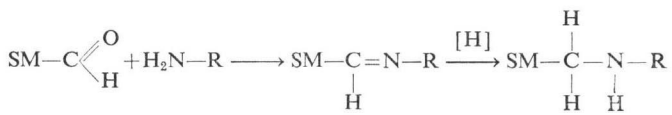
A homologue series of aliphatic streptomycylamines (SM-amines) have been prepared and tested *in vitro* (binding to 70S ribosomes) and *in vivo* (MIC). The short-chain SM-amines act as streptomycin (SM) but are less active than SM. They are inactive towards a SM-resistant *Escherichia coli*, our strain 042. The long-chain SM-amines are active both towards our sensitive and resistant *E. coli*, our strains 079 and 042. Their activities are not pH-dependent in contrast to that of SM. However, the higher homologues of the aliphatic amines (C_{10}~C_{16}) are considerably active *per se* although two to four times less than the corresponding SM-amines. Further, the amines do not compete with the SM-amine for the binding to the ribosomal particles. The binding affinities of these long-chain SM-amines to ribosomes are considerably smaller than that of SM. The binding is however specific as a typical isotope dilution curve can be obtained. We conclude that the long-chain SM-amines have a mode of action different from that of SM.

The antibacterial activity of streptomycin (SM) is dependent on the simultaneous presence of at least one guanidine group, the N-methyl-amino group and the aldehyde group of the streptose moiety. Structural modifications usually lead to a considerable loss of activity. Concerning the aldehyde group, which in aqueous solution is hydrated,[1] oxidation to a carboxyl group leads to complete inactivation.[2] Reduction of the diethyl-thioacetal with RANEY nickel to a methyl group leads to a loss of about 90 % of the activity,[3] whereas mild reduction (H_2, Pd) yields dihydrostreptomycin (DHS) which is fully active.[4] Condensation products with nitroalkanes preserve considerable activity,[5] whereas the oxime is devoid of activity.[6]

The streptomycylamines (SM-amines) are a class of related derivatives first described by WINSTEN & EIGEN.[7] They are prepared by condensing the aldehyde group of SM with primary amines followed by a reduction of the resulting SCHIFF-bases:



The antimicrobial activity of these compounds has been investigated by WINSTEN *et al.*[8] and by TREFFERS & ALEXANDER.[9] The main conclusion of their results is, that the higher homologues (C>6) are active both against SM-sensitive and SM-resistent strains of *E. coli*. The short chain homologues were active only towards SM-sensitive strains.

The biochemical explanation of the resistance phenomena was not elucidated at the time of these investigations. Resistance of an originally sensitive bacteria can be due to a phenotypic change in one of the ribosomal proteins as first demonstrated by the work of NOMURA and

coworkers[10] but strongly indicated already in 1961 by SPOTTS and STANIER.[11]

Resistance caused by plasmid mediated enzymatic inactivation (phosphorylation or adenylation of the 3'OH group) was demonstrated by OZANNE *et al.*[12] and UMEZAWA *et al.*[13] This type of resistance has become of increasing clinical importance. Among SM-resistant gram-negative bacteria it is assumed that more than half of these are of the inactivation types, and that the number is increasing rapidly (J. BANG, personal communication 1974).

In the light of this we wish to report on the *in vitro* activity of the SM-amines expressed by their binding affinities to the 70-S ribosomes compared to their *in vivo* activity.

Materials and Methods

Preparation of SM-amines

SM-trihydrochloride was dissolved in methanol (0.01 mol in 50 ml) to which was added 0.02 mol of the appropriate amine. The SCHIFF-base formation was facilitated by addition of 5 g of a molecular sieve (3A perlform, E. Merck, Darmstads, BRD). After standing overnight the solution was decanted from the molecular sieve, cooled on ice to 4°C and hydrogenated with $NaBH_4$ added in small portions. TLC and high voltage electrophoresis[14,5] showed that addition of approximately twice the calculated amount of $NaBH_4$ in all preparations performed lead to complete reduction. SM and DHS could not be detected, the detection limit was about 5%.

After reduction a large excess of triethylamine was added to the solution and poured into 8 volumes of acetone. The precipitates were redissolved in methanol and precipitated again. This procedure was repeated twice. The precipitates were finally dissolved in methanol containing 5 % of H_2O and the pH was adjusted to 6.0 with aqueous HCl. The tetrahydrochlorides of the SM-amines were isolated by precipitation with acetone, washed and dried. The yields were in all cases above 60 %. The ash content varied from 0 to 1 % and was considered of no importance for the biological experiments.

Using $NaB(^3H)_4$ (Amersham, Bucks, G. B.) in the reduction step T-DHS, T-butyl-SM-amine and T-dodecyl-SM-amine were prepared as described above. The specific activities of the three compounds were 4.9, 15.6 and 9.3 mCi/nmol.

SM-amine ($SM\text{-}CH_2NH_2$) was prepared as described in the literature.[15]

Growth of *E. coli* and preparation of ribosomes

Our *E. coli* strain 079 was grown in a 100-liter fermentor with aeration and stirring on a medium containing 3 g beef extract, 5 g tryptone and 10 g glucose per liter to a cell concentration corresponding to $OD_{525}=0.8$. The cells were harvested by centrifugation over a Sharples centrifuge (type MV-12-61Y-IJY), washed twice with buffer as described by NIRENBERG[16] and stored at −40°C. Ribosomes were prepared as described in the same publication.[16]

Binding experiments

These were performed as described earlier[17] with a minor modification. The radioactivity of the drug-ribosome complex isolated by Millipore filtration was measured directly in a toluene base liquid scintillation cocktail after drying of the filters.

In vivo activities

The *in vivo* activities of all compounds were estimated as Minimal Inhibitory Concentrations (MIC-values) in liquid nutrient broth using the conventional two fold dilution method.

Results and Discussion

We concentrated our studies on aliphatic straight chain SM-amines. The Rf (TLC) values including that of the common degradation product streptidine are shown in Table 1.

It is evident that increasing the lipophility of the derivatives decreases the Rf values

Table 1. Rf-values for SM-amines measured relative to DHS by TLC on silicagel plates as described by HEDING.[14]

Derivative	Rf
DHS	1.00
Streptidine	1.22
SM-amine	1.00
Ethyl-SM-amine	0.58
Propyl-SM-amine	0.42
Butyl-SM-amine	0.33
Hexyl-SM-amine	0.27
Decyl-SM-amine	0.15
Undecyl-SM-amine	0.12
Dodecyl-SM-amine	0.10
Hexadecyl-SM-amine	0.04

Table 2. Minimal inhibitory concentrations (MIC) of SM-amines and dodecylamine towards a sensitive *E. coli*, strain 079.

Derivative	MIC (μg base/ml)		
	pH 6.0	pH 6.8	pH 8.4
DHS	2	4	0.1
SM-amine	8	63	0.4
Ethyl-SM-amine	31	16	0.8
Propyl-SM-amine	16	16	0.8
Butyl-SM-amine	63	16	0.8
Hexyl-SM-amine	125	500	8
Decyl-SM-amine	4	4	1.6
Undecyl-SM-amine	4	4	3.2
Dodecyl-SM-amine	2	4	3.2
Hexadecyl-SM-amine	2	8	1.6
Dodecylamine	63	8	4

Table 3. Minimal inhibitory concentrations (MIC) of amines and mixtures of amines and DHS towards a sensitive *E. coli*, strain 079, at pH 6.8. MIC for DHS=4μg/ml.

Amine	MIC (μg/ml)	
	alone	in mixture with DHS
Ethylamine	125	8*
Propylamine	500	4
Butylamine	500	4
Hexylamine	500	4
Decylamine	31	8
Undecylamine	8	4
Dodecylamine	8	4
Hexadecylamine	4	4

* A MIC-value of 8μg/ml equals 8μg amine plus 8μg DHS base.

dramatically. Table 2 shows that SM-amines with carbon chains up to C_6 with respect to pH-dependence of activity exhibited the classical pattern described by EAGLE *et al.* in 1952.[19] This indicates that these compounds as antibiotics have a mode of action similar to that of SM. The very low activity of the hexyl-SM-amine was reexamined but was consistantly found to be low.

With carbon chains from C_{10} to C_{16} the derivatives showed no or only very little pH-dependence of the activity. The activities are however not significantly different from that of the amines compared in μg/ml as shown in Table 3. On a molar basis the SM-amines are significantly more active than the free amines. A possible synergistic effect between DHS and the long-chain amines could not be demonstrated.

The activities of SM-amines and the free amines towards an *E. coli* (042) made resistant by repeated transfer on SM-containing agar-substrate are illustrated in Table 4. The long-chain free amines are active but the corresponding SM-amines are on a molar basis a factor two to four times more active.

The activity of dodecylamine was measured at different pH-values towards the sensitive strain. The results are shown in Table 2, and it is evident that at pH 6.0 the dodecyl-SM-amine are several fold more active than the amine, also on weight basis.

The ability to compete with T-DHS for the SM-binding sites on the 70-S ribosomes was estimated, and the isotope dilution curves obtained are shown in Fig. 1. The compounds investigated fall into two groups: The short-chain SM-amines, including streptomycylamine, have a lower affinity to the ribosomes than DHS in good agreement with their lower *in vivo*

Table 4. Minimal inhibitory concentrations (MIC) of SM-amines and amines towards a resistant *E. coli*, strain 042, at pH 6.8.

SM-amines	MIC (μg base/ml)	Amine	MIC (μg/ml)
DHS	1,000		
SM-amines	500		
Ethyl-SM-amines	500	Ethylamine	500
Propyl-SM-amines	500	Propylamine	1,000
Butyl-SM-amines	500	Butylamine	1,000
Hexyl-SM-amines	63	Hexylamine	1,000
Decyl-SM-amines	4	Decylamine	16
Undecyl-SM-amines	2	Undecylamine	8
Dodecyl-SM-amines	4	Dodecylamine	4
Hexadecyl-SM-amines	2	Hexadecylamine	4

activity. The higher homologues (C_{10}, C_{11}, C_{12} and C_{16}) do not compete with the binding of DHS although they have considerable antimicrobial activity. Similarily hexyl-SM-amine does not compete with DHS for the binding sites, but this compound is almost devoid of *in vivo* activity (Table 2).

In higher concentrations the dodecyl-SM-amine and the butyl-SM-amine exhibit a specific binding expressed by the isotope dilution curves shown in Fig. 2a and 2b. These curves also show that the unsubstituted amines, butylamine and dodecylamine, do not compete with the SM-derivatives for their binding sites. The antimicrobial activity of dodecylamine must be due to some other interaction with the bacteria.

It can be concluded that the SM-amines with a short-chain aminosubstituent acts as streptomycins whereas the SM-amines with long-chain aminosubstituents ($C_{10}\sim C_{16}$) are antibiotics with a different mode of action, which renders them active towards SM-sensitive as well as a SM-

Fig. 1. Isotope dilution curves.
T-DHS was incubated with increasing amounts of SM-derivatives in the presence of 70-S particles. The amount of bound T-DHS was measured.
1: DHS 6: Hexyl-SM-amine
2: SM-amine 7: Decyl-SM-amine
3: Ethyl-SM-amine 8: Undecyl-SM-amine
4: Propyl-SM-amine 9: Dodecyl-SM-amine
5: Butyl-SM-amine 10: Hexadecyl-SM-amine

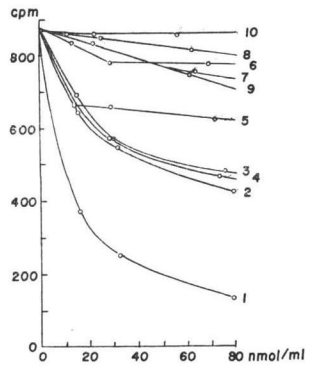
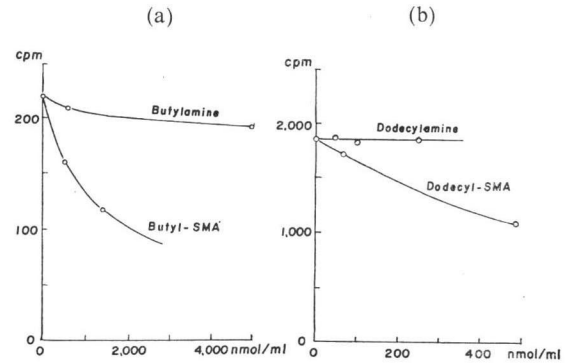


Fig. 2. Isotope dilution curves.
(a) T-butyl-SM-amine was incubated with increasing amounts of butyl-SM-amine and butylamine in the presence of 70-S particles. The amount of bound T-butyl-SM-amine was measured.
(b) T-dodecyl-SM-amine was incubated with increasing amounts of dodecyl-SM-amine and dodecylamine in the presence of 70-S particles. The amount of bound T-dodecyl-SM-amine was measured.



resistant (ribosomal) *E. coli*. The activities of these compounds can not be attributed to the aminosubstituent *per se* as these do not compete with the binding of the SM-derivatives to the ribosomes.

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