SEMI-SYNTHESIS OF A23187 (CALCIMYCIN) ANALOGS

IV. CATION CARRIER PROPERTIES IN MITOCHONDRIA OF ANALOGS WITH MODIFIED BENZOXAZOLE RINGS. ANTIMICROBIAL ACTIVITY

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The transporting abilities in the mitochondrial membrane for Ca^{++} and Mg^{++} of ten semi-synthetic analogs A23187 (calcimycin) and X14885A are compared. Analogs classified as efficient divalent cation carrier retained the calcimycin antimicrobial activity against three Gram-positive strains tested.

In a previous paper[1], we described a semi-synthetic approach to obtain various calcimycin analogs bearing a modified benzoxazole moiety. Further, we studied their transporting abilities for calcium and magnesium through an organic phase. In order to compare the physico-chemical model with a relevant biological membrane, a study has been performed in rat liver mitochondria which has remained a valuable test system for the newer ionophores[2]. Analogs studied are mentioned in Table 1.

Release of Calcium and Magnesium in Rat Liver Mitochondria

The experiments were carried out following a previously reported method[3]. The mitochondria were incubated with 200 mM sucrose, 20 mM HEPES - NaOH (pH 7.4~7.6), 5 mM glutamate, in the presence of increasing amounts of ionophore. Reaction was stopped within 1 minute by rapid centrifugation. Longer incubation was not necessary. The divalent cation content of the pellet was determined by atomic absorption. The percentage of endogeneous calcium and magnesium released was plotted as a function of the ionophore concentration (in nmol/mg protein) for each analog (Fig. 1).

Of the molecules which were able to release efficiently the divalent cations, calcimycin was the best; less than 1 nmol/mg protein induced 75% of depletion in calcium and 70% in magnesium, after which the sequence was **5**>**3**~**7**~**12**>**4**. Sterically hindered compounds **6**, **8** to **11** stood out clearly. Interestingly, for all the good carriers, amounts of calcium released were higher than those of magnesium. This was reversed for poor transporting systems probably because they cannot compete with the mitochondrial calcium transport[4] which is not blocked.

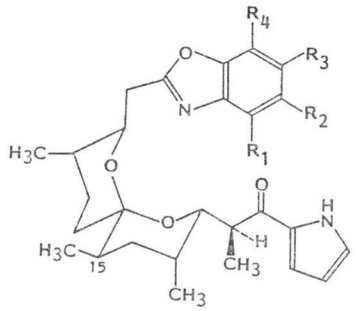
The mitochondria test does not allow a strict sequence to be established. However, all the analogs classified as good extracting systems in the organic phase[1], were able to release significantly calcium and magnesium in the mitochondrial membrane. As a final investigation we measured the *in vitro* inhibition of growth for some microorganisms.

Antibacterial Activity

The minimum inhibitory concentration (in μg/ml) recorded in Table 2, were determined by the broth dilution test on five strains.

Although the responses were not strictly identical from one strain to another, compounds classified as efficient ionophorous systems in the previous experiments retained a large part of the parent activity

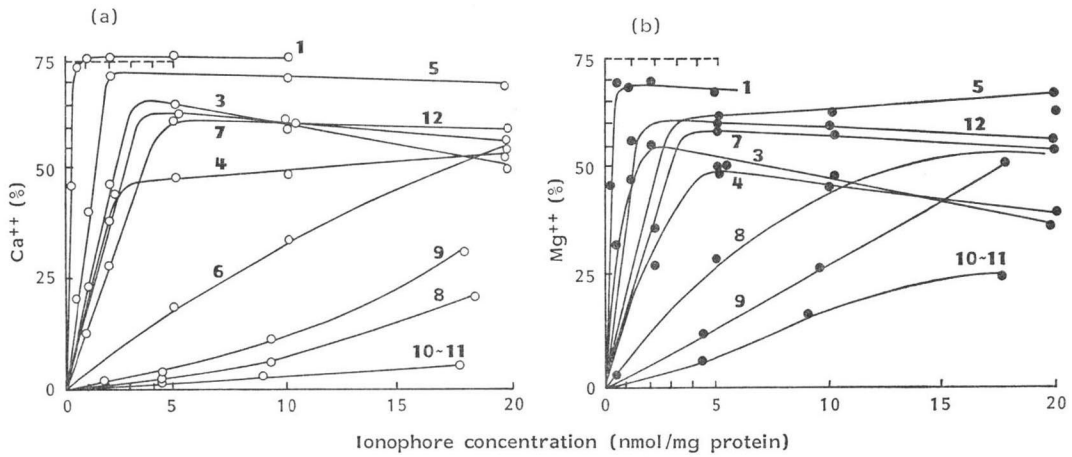
Table 1. Analogs studied.



Compounds	R_1	R_2	R_3	R_4
1	COOH	$NHCH_3$	H	H
2	H	COOH	H	H
3	COOH	H	H	H
4	COOH	H	H	CH_3
5	COOH	H	CH_3	H
6	COOH	CH_3	H	H
7	COOH	OH	H	H
8	COOH	$N(CH_3)_2$	H	H
9	COOH	$N(CH_3)C_2H_5$	H	H
10	COOH	$N(CH_3)COCH_3$	H	H
11	COOH	$N(CH_3)COCF_3$	H	H
12*	COOH	OH	H	H

* X14885A: Backbone without 15-methyl.

Fig. 1. Calcium and magnesium efflux in rat liver mitochondria.
Percentage of cation released versus ionophore concentration.



against the three Gram-positive bacteria and likewise showed a very low activity against *Streptomyces* and the *Penicillium* strains. Interestingly, the semi-synthetic compound **7** appeared to have the best activity, higher even than **1**. Compounds with a bulky R_2 substituent were largely inactivated. This was still more pronounced for **2** where the COOH group is in the *meta* position relative to the oxazolic nitrogen.

A strict relationship between the transporting abilities and the MIC results cannot be established,

Table 2. *In vitro* antimicrobial activities by broth dilution tests (MIC μg/ml).

Compounds	*Bacillus cereus* ATCC 14575	*Bacillus megaterium* ATCC 14581	*Micrococcus luteus* ATCC 4698	*Streptomyces rimosus* NRRL 2234	*Penicillium decumbens* NRRL 742
1	0.024	0.0015	0.012	50	12.5
2	25	6.25	12.5	50	50
3	0.097	0.049	0.195	3.12	25
4	0.39	<0.0015	0.006	25	6.25
5	0.39	0.024	0.012	50	12.5
6	6.25		0.78	50	25
7	0.006	<0.0015	0.024	50	100
X14885A (**12**)	0.097	0.006	0.006	6.25	50
8	3.12	0.39	0.39	25	50
9	1.56	1.56	3.12	50	50
10	12.5	6.25	12.5	50	50
11	0.78	0.20	0.78	12.5	25

but it is noteworthy that the antibiotic activity is retained along with the ionophorous properties. Any finer interpretation would need a better understanding of the antibacterial mechanism of action for A23187.

It has been proposed that the inhibition effect is probably due to a loss of Mg^{++} from the cell[5]. Very recently, ALATOSSAVA[6] showed that an A23187 concentration of 3 μg/ml is enough to induce complete Mg^{++} depletion of the *Lactobacillus lactis* cell (about a 9-fold decrease). There is thus an interesting convergence between those two pieces of work and the results given in Table 2, but a concomitant uptake of $2H^+$ should take place, and so the effect on the intracellular pH might also play a major role in this context. As stressed above, calcimycin (and analogs) are preferentially calcium rather than magnesium carriers, so one may wonder for what purpose this secondary metabolite is produced by the *Streptomyces chartreusis* strain. This is still an open question.

In summary, our work focuses on the A23187 benzoxazole ring and its implication for the ionophorous properties of the molecule. We have shown that the two major liganding sites, the carboxylic group and the pyridine-like oxazole nitrogen, must be located in the *ortho* position for complex stability and the ensuing biological activity. Further, the carboxylic group must stay coplanar with the aromatic ring, otherwise the coordination sphere around the divalent cation is disturbed and the 2:1 associations destabilized. In the latter case, the transporting abilities in the mitochondrial membrane are almost completely lost as is the antibiotic activity.

Recent work has shown that the polar benzoxazole group is located at the water-membrane interface in either protonation state[7]. One can thus assume that the $NHCH_3$ group (or OH in X14885A) holds the carboxylic group in a favorable position, especially in the anionic form. In the transporting process, the capture of the cation, which is most probably initiated in that part of the molecule during the complex formation at the interface is thereby facilitated. Besides, *ab initio* calculations carried out recently on the calcimycin-magnesium complex have shown that the secondary amine group provides an additional stabilizing factor for the association by its electron-donating effect[8].

All these observations add weight to the importance of the role of the 2-carboxy-3-*N*-methylaminobenzoxazole ring in calcimycin. However, a semi-synthetic analog such as **5** (R_1=COOH, R_2=R_4=H, R_3=CH_3) has quite similar behavior to that of the natural ionophore. The synthetic approach described may therefore prove helpful in the design of new carriers with functionalized or labeled R_3,

for the purpose of carrying out specific biological investigations.

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