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**LYSOSOMOTROPIC N,N- DIMETHYL α -AMINOACID n-ALKYL
ESTERS AND THEIR QUATERNARY AMMONIUM SALTS AS
PLASMA MEMBRANE AND MITOCHONDRIAL ATPases
INHIBITORS**

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Abstract: A set of n-alkyl esters of N,N-dimethylglycine (DMG-n) and their methobromides (DMGM-n) was synthesized, and their activities on yeast *Saccharomyces cerevisiae* were compared. The compounds differ in the number of carbon atoms in the aliphatic chain. Aminoesters with 12 carbon atoms appeared to be most active. Unlike quaternary ammonium salts previously tested, the activities of the compounds were not pH-dependent; the minimal inhibitory concentrations (MIC) were identical at pH 8 and at pH 6. In contrast to quaternary ammonium salts, aminoesters showed similar effects on respiratory sufficient (ρ^+) and respiratory deficient (ρ^0) mutants.

When tested on glucose stimulated proton extrusion, aminoesters applied at MIC increased external pH. Aminoesters inhibited the plasma membrane H^+ -ATPase, whereas they were less inhibitory on the mitochondrial ATPase.

In order to further compare the aminoesters and their corresponding quaternary ammonium salts, derivatives of N,N-dimethylalanine (DMAL-n and DMALM-n, respectively) were synthesized. The quaternary ammonium salts appeared to have a higher inhibitory potency than aminoesters, especially at pH 8, and alanine derivatives inhibited growth at a lower concentration than glycine derivatives. Both alanine derivatives of the aminoester and the quaternary ammonium salt inhibited the plasma membrane H^+ -ATPase at lower concentrations than glycine derivatives, but the alanine aminoester was without a detectable effect on the mitochondrial ATPase.

Key Words: Quaternary Ammonium Salts, Lysosomotropic Aminoesters, H⁺-ATPase, *Saccharomyces cerevisiae*, Proton Extrusion

INTRODUCTION

The n-alkyl N,N-dimethylglycinates (DMG-n) and dimethylalaninates (DMAL-n) as well as their corresponding quaternary ammonium salts (DMGM-n and DMALM-n) synthesized in our laboratory were investigated in order to find a possible relationship between their chemical structure and biological activity on yeast *Saccharomyces cerevisiae*. The growth inhibitory activities of other quaternary ammonium salts and aminoesters were presented in previous publications [1-6]. The present paper further exemplifies the contribution of quaternary ammonium salts and lysosomotropic amines to inhibition of yeast.

MATERIALS AND METHODS

The compounds

The general structure of aminoesters and their corresponding quaternary ammonium salts is presented in Fig. 1. The compounds were synthesized in our laboratory by amination of n-alkyl chloroacetates or α-bromopropionates with dimethylamine in ethereal solution [7]. N,N-Dimethyl glycinates (DMG-n) and alaninates (DMAL-n) were purified by preparation of their hydrochlorides or succinates. Free esters of both aminoacids were quaternized by methyl bromide in ethereal solution to the corresponding methobromides of glycine (DMGM-n) and alanine (DMALM-n) derivatives. ¹H-NMR spectra (Brucker instrument 300MHz, CDCl₃, HMS as internal standard) confirmed the high purity of the synthesized compounds. Both the hydrochlorides of aminoesters and their quaternary ammonium salts are well soluble in water and were supplemented to the growth or test media at the indicated final concentrations.

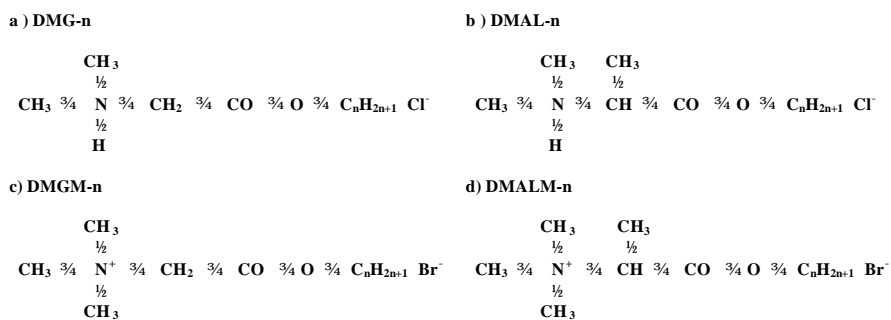


Fig.1. Chemical structure of the aminoesters: a) DMG-n (n-alkyl N,N-dimethyl-glycinates), b) DMAL-12 (n-alkyl N,N-dimethyl-alaninates) and their corresponding quaternary ammonium salts: c) DMGM-n, d) DMALM-n, where n=10,12,14,16.

Strain

Saccharomyces cerevisiae strain Σ 1278b was used as a test microorganism. Its respiratory deficient rho⁰ mutant was isolated by ethidium bromide treatment [8]. The yeast was grown in YPD medium (1% yeast extract Difco, 1% bacto peptone Difco, 2% glucose). For plating, the medium was supplemented with 2% bacto agar. Its pH (6 or 8) was adjusted by Sørensen phosphate buffer (0.05M).

Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration was determined as the lowest concentration of the compound that inhibits formation of the colonies on YPD plates spreader with 100-200 yeast cells of an overnight YPD liquid culture.

Proton extrusion test

Glucose induced proton extrusion tests were performed as described by Sigler *et al.* [9] and Kotyk [10].

ATPase activity assays

ATPase activity assays were done according to Dufour and Goffeau [11], using the modification of Ułaszewski [12]. The rate of ATP hydrolysis was determined by measuring the amount of released inorganic phosphate. The amount of protein was determined according to Lowry *et al.* [13], using bovine serum albumin as a standard.

RESULTS

The main difference in chemical structure between the quaternary ammonium salts (QAS-es), represented here by n-alkyl N,N-dimethylglycinate (DMGM-n) or alaninate (DMALM-n) methobromides, and the starting lysosomotropic aminoesters, is the polar "head". QAS-es are positively charged with a stable charge at the nitrogen atom, but aminoesters (DMG-n or DMAL-n) are in a pH-dependent dynamic equilibrium and a positive charge on the nitrogen atom is present only in their protonated form (Fig.1).

Both aminoesters (DMG-n) and their quaternary ammonium salts (DMGM-n) inhibit growth of baker's yeast *Saccharomyces cerevisiae* when present in the nutrient medium YPD. The growth inhibitory activity of aminoesters and of QAS-es is dependent on the carbon chain length, with an optimum observed at twelve carbon atoms. Only in the case of DMALM-14 the derivative containing a 14-carbon atom chain was shown to cause the same level of inhibition as the compound with a 12-carbon atom chain. It was also found that aminoesters with 16 carbon atoms in the aliphatic chain seem to be less active than the corresponding QAS-es (Tab. 1).

In the previous work [6] it was found that the inhibitory activity of QAS (IM) is pH-dependent. The MIC is lower at pH 8 than at pH 6. Thus the influence of pH

on MIC was tested for DMG-n and DMGM-n. Using alanine derivatives (DMAL-n) and their quaternary ammonium salts (DMALM-n) similar tests on the MIC value were performed, too. The results shown in Tab. 1 indicate that alanine derivatives as quaternary ammonium salts are more active than glycine derivatives, and that the MIC for the compounds, especially for QAS-es, is clearly pH-dependent; being at least five time lower at a higher pH.

The previously tested QAS (IM) showed a clear-cut difference in growth inhibition depending on the respiratory competence of yeast. The respiratory deficient mitochondrial mutants ρ^0 or ρ^- appeared to be more sensitive than the corresponding original respiratory competent ρ^+ form. This difference in sensitivity was observed both at pH 6 and pH 8. Therefore the influence of respiratory competence on the sensitivity to the glycine DMG-12, DMGM-12 and alanine DMAL-12, DMALM-12 derivatives was tested.

Tab. 1. pH-dependent minimal inhibitory concentrations (MIC) of glycine (DMG-n) and alanine (DMAL-n) aminoesters and their corresponding quaternary ammonium salts (DMGM-n and DMALM-n) for yeast *Saccharomyces cerevisiae* growth.

Compound	N° of carbon atoms	MIC (μ M) at pH	
		6	8
DMG-n	10	160	-
	12	40→80	40
	14	80	-
	16	>320	-
DMGM-n	10	160	40
	12	80	10
	14	160	20
	16	320	20
DMAL-n	10	160	80
	12	40	160
	14	320	>320
	16	>320	>320
DMALM-n	10	40	5
	12	5	1
	14	5	1
	16	10	2

(-) no data

As shown in Tab. 2, the MIC for the aminoesters DMG-12 or DMAL-12 appears to be less dependent on the respiratory competence of yeast at both pH tested. In contrast, the corresponding quaternary ammonium salts inhibit growth of the respiratory mutant cells (ρ^0) at a much lower concentration than the original ρ^+ cells at both pH values tested. pH 8 is rather physiologically unsuitable for yeast. Under these conditions, the growth rate of yeast cells is reduced. Therefore, the plates had to be incubated for at least 7 days before scoring.

The aminoesters (DMG-12 or DMAL-12) differ from their corresponding quaternary ammonium salts (DMGM-12 or DMALM-12) in their inhibitory potency to inhibit the plasma membrane H⁺-ATPase and the mitochondrial ATPase. The I₅₀ for the plasma membrane H⁺-ATPase is rather low and alanine derivatives are more active than glycine derivatives. With the mitochondrial ATPase, aminoesters showed an inhibitory effect at higher concentrations or were not inhibitory at all (Tab. 3).

Tab. 2. Influence of respiratory competence of yeast *Saccharomyces cerevisiae* (rho⁺ and rho^o) on sensitivities to aminoesters (DMG-12 and DMAL-12) and their corresponding quaternary ammonium salts (DMGM-12 and DMALM-12). MIC was determined as described in Materials and Methods.

Compound	pH	MIC (μM) for	
		rho ⁺	rho ^o
DMG-12	6	40→80	40→80
	8	40	40
DMGM-12	6	80	10
	8	10	<5
DMAL-12	6	40	40
	8	160	160
DMALM-12	6	5	1
	8	1	<1
DMALM-14	6	5	1
	8	1	<1

Tab. 3. Effect of glycine and alanine derivatives on yeast plasma membrane H⁺-ATPase and mitochondrial ATPase activities.

Derivative	I ₅₀ [*] (μM) for	
	plasma membrane H ⁺ -ATPase	mitochondrial ATPase
DMG-12	20	230
DMGM-12	29	27
DMAL-12	8	no inhibition
DMALM-12	12	18
DMALM-14	8	11

I₅₀^{*} – drug concentration that inhibits 50 per cent of the enzyme activity

The assay for glucose stimulated proton extrusion demonstrated that quaternary ammonium salts had a higher inhibitory potency than aminoesters. For example,

DMG-12 at 80 μM inhibits proton extrusion followed by a slight increase in external pH. The corresponding quaternary ammonium salt (DMGM-12) used at the same concentration causes an abrupt increase in pH (Fig.2a and 2b). Similar results were obtained with alanine derivatives (not shown).

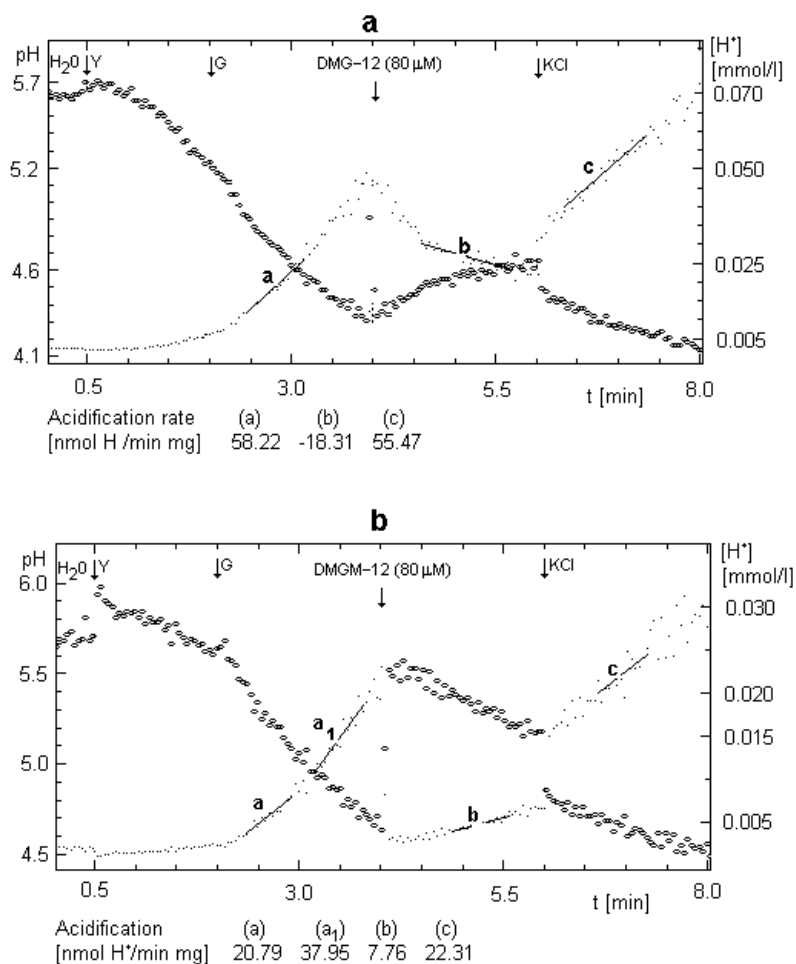


Fig. 2. The inhibition of glucose stimulated proton extrusion by DMG-12 or DMGM-12 in the original strain $\Sigma 1278b$: **a**, 80 μM DMG-12, **b**, 80 μM DMGM-12. Arrows indicate successive addition (to final concentrations) of: Y – yeast cells (2 mg of dry weight), G – glucose (200 mM), DMG-12 or DMGM-12 (80 μM), KCl (100 mM). Heavy-dotted line - pH, fine-dotted line (a and a₁, b, c) – [H⁺]. Addition of KCl causes acidification reconstituted by K⁺.

DISCUSSION

The biological activity of aminoesters as well as their corresponding quaternary ammonium salts on yeast depends on the length of the aliphatic chain with an optimum at 12 carbon atoms. This consistent pattern is essentially also applicable to glycine and alanine aminoesters. We do not have experimental results that allow us to explain this pattern, but a similar maximum is observed in several types of cationic surfactants [7,14,15].

A higher inhibitory potency of quaternary ammonium salts compared to aminoesters is the second observed pattern. This was noticed with both glycine and alanine derivatives. This difference can be explained by the presence of a charge on the polar "head" containing nitrogen, which is strongly positive in quaternary ammonium salts. This charge should make it difficult for quaternary salts to pass through the plasma membrane. In fact, quaternary ammonium salts anchor on the surface of plasma membranes, thus interfering with its function, especially with transport.

It is supposed that aminoesters are not stopped on the outside surface of the plasma membrane, but penetrate cells, where they undergo protonation either in the cytoplasm or in the intracellular compartments, and acquire their biological activity.

The third characteristic feature of quaternary ammonium salts of glycine and alanine derivatives is the dependence of their biological activity on the medium pH. In a slightly alkaline environment (pH 8), their MIC is considerably lower than at pH 6. The simplest interpretation of these observations can be connected with the fact that a neutral or slightly alkaline environment is unsuitable for yeast. Thus, the increase in the pH of the medium causes an additional stress on yeast cells, in particular in the presence of quaternary ammonium salts. However, this pH-dependence is not observed with aminoesters, which would suggest a difference in the mechanism of action of these two groups of compounds.

This is supported by the difference in the inhibitory potency between strains with respiratory deficiency (ρ^0) and control strains (ρ^+).

The mitochondrial mutants with a respiratory defect are much more sensitive to quaternary ammonium salts than the original respiratory proficient strain. This difference is not observed with aminoesters. We hypothesized that quaternary ammonium salts are inhibitors of active amino acid transport into the cells, which had previously found experimental confirmations [1,5,16]. The active transport requires an input of energy (ATP). The efficiency of ATP production in respiratory deficient mutants is clearly lower than in respiratory proficient cells. Hence the decreased uptake of the amino acid should manifest itself by an increased sensitivity to quaternary ammonium salts.

Finally, the difference in the mode of action between quaternary ammonium salts and aminoesters points to their effect on the yeast plasma membrane ATPase and the mitochondrial ATPase. The plasma membrane ATPase is

inhibited by both quaternary ammonium salts and aminoesters. On the other hand, the mitochondrial ATPase is inhibited by quaternary ammonium salts and is only slightly sensitive or entirely insensitive to aminoesters. This may be explained by the fact that at a higher pH, at which the mitochondrial ATPase activity was tested, aminoesters are mainly in the form of free (uncharged) compounds. Thus, it appears that free aminoesters do not interact with the ATPase and the slight inhibition may arise from the charged protonated form in the equilibrium mixture present as a minor component.

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REFERENCES

1. Lachowicz T.M., Obląk E. and Piątkowski J. Auxotrophy – stimulated sensitivity to quaternary ammonium salts and its relation to active transport in yeast. **Bul. Pol. Acad. Sci.** 40 (1992) 173-182.
2. Obląk E., Ułaszewski S., Morawiecki A., Witek S., Witkowska R., Majcher K. and Lachowicz T.M. Quaternary ammonium salt resistant mutants in yeast *Saccharomyces cerevisiae*. **Yeast** 5 (Special Issue) (1989) 273-278.
3. Obląk E., Ułaszewski S. and Lachowicz T.M. Mutants of *Saccharomyces cerevisiae* resistant to a quaternary ammonium salt. **Acta Microbiol. Polon.** 37 (1988) 261- 269.
4. Lachowicz T. M., Witkowska R. and Obląk E. Amino acid auxotrophy increases sensitivity of *Saccharomyces cerevisiae* to a quaternary ammonium salt IM. **Acta Microbiol. Polon.** 39 (1990) 157-162.
5. Obląk E., Bącal J. and Lachowicz T.M. A quaternary ammonium salt as an inhibitor of plasma membrane H⁺-ATPase in yeast *Saccharomyces cerevisiae*. **Cell. Biol. Mol. Lett.** 5 (2000) 315 – 324.
6. Obląk E., Lachowicz T.M., Luczyński J.m and Witek S. Comparative studies of biological activities of the lysosomotropic aminoesters and quaternary ammonium salts on yeast *Saccharomyces cerevisiae*. **Cell. Biol. Mol. Lett.** 6 (2001) 871 – 880.
7. Thompson R., Amphiphilic glycine-based esters as soft antimicrobial agents, **PhD Thesis**, Goeteborg 1992
8. Słonimski P.P., Perodin G. and Crought J.H. Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient nonchromosomal “petites”. **Biochem. Biophys. Res. Commun.** 30 (1968) 232.
9. Sigler K., Knotkova A. and Kotyk A. Factors governing substrate induced generation and extrusion of protons in the yeast *Saccharomyces cerevisiae*. **Biochim. Biophys. Acta.** 643 (1981) 572-582.
10. Kotyk A. Mechanisms of extracellular acidification by yeast. **J. Biophys.** 3 (1993) 17-26.

11. Dufour J.P. and Goffeau A. Solubilization by lysolecithin and purification of the plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. **J. Biol. Chem.** 253 (1978) 7026-7032.
12. Ułaszewski S. Interactions between the gene products of pam1 encoding plasma membrane H⁺-ATPase, and pdr1 controlling multiple drug resistance in *Saccharomyces cerevisiae*. **Acta Biochim. Polon.** 40 (1993) 487-496.
13. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. Protein measurement with the Folin phenol reagent. **J. Biol. Chem.** 193 (1951) 265-275.
14. Górska-Poczopko J. and Witek S. Fungicidal properties of some new quaternary ammonium salts. **Abh. Akad. Wiss. Deutch. (Berlin)** 1 (1982) 271-275.
15. Rucka M., Oświęcimska M. and Witek S. New biocides for cooling water treatment. III Quaternary ammonium salts derivatives of glycine esters. **Envir. Protect. Eng.** 9 No. 3 (1983) 25-31.
16. Obłąk E., Lachowicz T.M. and Witek S. DL - leucine transport in a *Saccharomyces cerevisiae* mutant resistant to quaternary ammonium salts. **Folia Microbiol.** 41 (1996) 116-119.