

A Semisynthetic 5-*n*-Alkylresorcinol Derivative and its Effect upon Biomembrane Properties

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MSAR (1-sulfate-3-myristoyl-5-pentadecylbenzene) is a semisynthetic derivative of 5-*n*-pentadecylresorcinol (C15:0). MSAR exhibits hemolytic activity against sheep erythrocytes with a EH_{50} value of $(35 \pm 1.7) \mu\text{M}$. At low concentrations MSAR also exhibits the ability to protect cells against their hypoosmotic lysis. This protective effect is significant as, at $0.1 \mu\text{M}$ of MSAR, the extent of osmotically induced cell lysis is reduced by approx. 20%. It was demonstrated that the 9-anthroxystearic acid signal was most intensively quenched by MSAR molecules, suggesting a relatively deep location of these molecules within the lipid bilayer. MSAR causes an increase of the fluorescence of the membrane potential sensitive probe. This indicates an alteration of the surface charge and a decrease of the local pH value at the membrane surface. At low bilayer content (1–4 mol%) this compound causes a significant increase of the phospholipid bilayer fluidity (both under and above the main phase transition temperature) of dipalmitoylphosphatidylcholine (DPPC) liposomes. At this low content MSAR slightly decreases the main phase transition temperature (T_c) value. The effects induced in the phospholipid bilayer by higher contents of MSAR molecules (5–10 mol%) make it impossible to determine the T_c value and to evaluate changes of the membrane fluidity by using pyrene-labeled lipid. MSAR also causes a decrease of the activity of membrane-bound enzymes – red blood cell acetylcholinesterase (AChE) and phospholipase A_2 (PLA₂). MSAR decreases the AChE activity by 40% at $100 \mu\text{M}$. The presence of MSAR in the liposomal membrane induces a complete abolishment of the lag time of the PLA₂ activity, indicating that these molecules induce the formation of packing defects in the bilayer which may result from imperfect mixing of phospholipids.

Key words: Phenolic Lipids, Hemolytic Activity, Phospholipase A_2 , Acetylcholinesterase

Introduction

MSAR (1-sulfate-3-myristoyl-5-pentadecylbenzene) is a semisynthetic derivative of 5-*n*-pentadecylresorcinol (C15:0), one of the members of resorcinolic lipids. Resorcinolic lipids, the natural amphiphilic long-chain homologues of orcinol (1,3-dihydroxy-5-methylbenzene), were demonstrated in numerous plants (Wenkert *et al.*, 1964; Wieringa, 1967), microbial (Batrakov *et al.*, 1977; Bu'Lock and Hudson, 1969; Kozubek *et al.*, 1996) and fungal organisms (Giannetti *et al.*, 1978). They exhibit a strong amphiphilic character with values of the octanol/water partition coefficient ($\log P_{o/w}$)

over 7.4 (Kozubek, 1995) and show high affinity for lipid bilayers as well as for biological membranes. The incorporation of homologues into liposomal and biological membranes induces an increase of their permeability for small non-electrolytes and cations (Kozubek and Demel, 1980; Kozubek, 1987). This increase of the permeability of membranes may result in the formation of the non-bilayer structures within the bilayer, such as reversed micelles or reversed hexagonal phase (H_{II}) (Kozubek and Demel, 1981) and often results in the hemolysis of the cells.

Previous studies on MSAR showed that this semisynthetic lipid forms stable liposomal struc-

tures in buffered conditions by itself as well as in mixtures with phospholipids and sterols in any molar ratio (Gubernator *et al.*, 2001). In the present study we focused on the characterization of the biological properties of MSAR as an amphiphilic molecule and its effect on the biological membranes and the activity of membrane enzymes.

Materials and Methods

Materials

The fluorescent probes: fluorescein-DHPE, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β -py-C₁₀-HPC), 1,2-bis-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (BPHPC) and various types of AS (9-anthroxystearic acid) were purchased from Molecular Probes (Eugene, OR, USA). Egg yolk phosphatidylcholine (PC) and phospholipase A₂ (PLA₂) from *Naja mocambique mocambique* venom were obtained from Sigma-Aldrich (Poznan, Poland) and dipalmitoylphosphatidylcholine (DPPC) from Lipids Products (Nutfield, Surrey, GB). Other chemicals were of the best available purity from POCh (Gliwice, Poland).

Methods

The methodological details are kept at the author's desk. Interested readers should contact the authors directly for information.

Results and Discussion

Hemolytic and antihemolytic activity of MSAR

MSAR (Fig. 1) present in the incubation medium exhibits hemolytic activity against sheep erythrocytes with an EH₅₀ value of (35 ± 1.7) μM. This value is similar to that demonstrated for 5-*n*-pentadecylresorcinol [EH₅₀ (37.5 ± 2.5) μM] (Stasiuk and Kozubek, 1996, 1997a). Almost identical values of EH₅₀ suggest that the presence of a negatively charged substituent (sulfate) affects the ability of the hydrophobic part of the molecule to disrupt the membrane barrier properties. This polar substituent seems to be responsible for the differences in the solubility of MSAR [critical micelles concentration (CMC) (0.893 ± 0.5) mM]

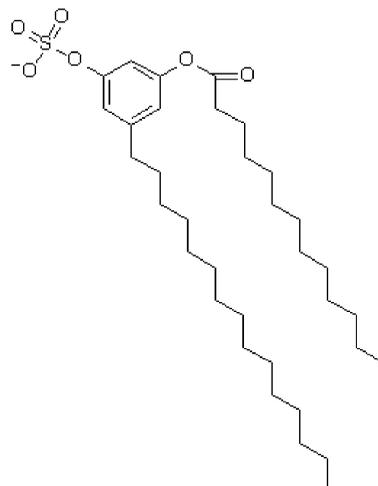


Fig. 1. The structure of MSAR (1-sulfate-3-myristoyl-5-pentadecylbenzene).

and pentadecylresorcinol [CMC (2.65 ± 0.6) μM (Stasiuk and Kozubek, 1998)] in aqueous solutions and for the differences in the molecular shape of the molecule and its electric charge. Having two aliphatic chains MSAR exhibits, similar to pentadecylresorcinol, the ability to incorporate into the erythrocyte membrane, but its solubility in aqueous solutions is significantly higher most probably due to the significantly stronger hydrophilicity of the sulfate polar head. It should be noted that the methods used for the CMC determination of MSAR and AR (alkylresorcinol) C15:0 were different as the spectrophotometrical method did not work well in case of AR C15:0, because its CMC value was determined by surface pressure changes (Devinsky *et al.*, 1985).

MSAR at low concentration, well below that inducing lysis of erythrocytes, also exhibits the ability to protect cells against their lysis in hypoosmotic solutions (Fig. 2). This protective effect is significant at 0.1 μM of MSAR, where the extent of osmotically induced cell lysis is reduced by approx. 20%. Considering the lowest concentration of the compound at which it stabilizes the erythrocyte membrane in hypoosmotic solution, MSAR is more active than other phenolic lipids, *e.g.* anacardic acid protects erythrocytes against hypoosmotic lysis only at 10% (at higher concentration – 1 μM) and merulinic acid protects erythrocytes at 40% (at even higher concentration – 10 mM) (Stasiuk *et al.*, 2004). This activity of MSAR, called “antihemolytic”, is similar to that demonstrated for cardol

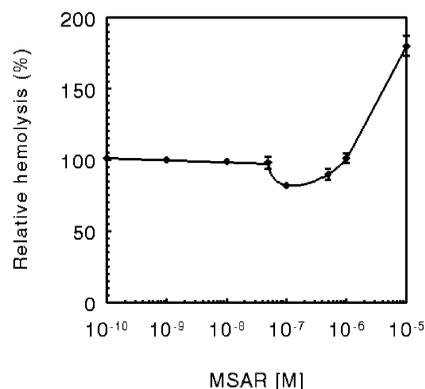


Fig. 2. The antihemolytic effect of MSAR upon sheep erythrocytes. Data are given as means \pm SD ($n = 3-4$).

[5-*n*-pentadec(en)ylresorcinol] which at the concentration of $10 \mu\text{M}$ inhibits 50% of hypoosmotically induced lysis of erythrocytes (Stasiuk and Kozubek, 1997b).

Effect of MSAR upon liposomal surface charge and mobility of lipids

The effect of MSAR upon bilayer properties was also demonstrated when its effect upon the surface charge and local pH value was studied. Fluorescein-PE is a fluorescent probe that is used as a membrane label or a sensor of surface-associated processes. Fluorescein-PE fluorescence intensity depends not only on the bulk pH value, but also on the local electrostatic potential, which affects the local membrane interface proton concentration. The fluorescence activity of this probe depends on the dissociation of the COOH group, located near the lipid membrane surface, and the temperature. The fluorescence of the probe decreases with increasing pH value (Langner *et al.*, 2000). The MSAR molecule contains an ionizable $-\text{SO}_3^-$ group, that caused an increase of the fluorescence of the membrane probe from 2% (at 1 mol%) to approx. 8% (at 17 mol%). This indicated a decrease of the local pH value at the membrane surface. On the other hand, experiments concerning the effect of the studied compound upon the properties of the bilayer across its thickness, as monitored by the alteration of the fluorescence behaviour of probes localized at various depths of the bilayer (NBD-PE in the hydrophilic region and TMA-DPH in the deeper region, below C4 of the acyl chains of phospholipids), did

not show any visible effect (Fig. 3). Because of this fact studies of quenching of *n*-AS by MSAR were performed. The appropriate series of *n*-AS were chosen for this study which are localized at a defined depth of the bilayer and it was already shown that phenolic derivatives (*e.g.* local anesthetic tetracaine) exhibited quenching properties against *n*-AS dyes (Hutterer *et al.*, 1997). In this experiment MSAR was added externally to the suspension of preformed liposomes to see how this compound affects the bilayer during incorpora-

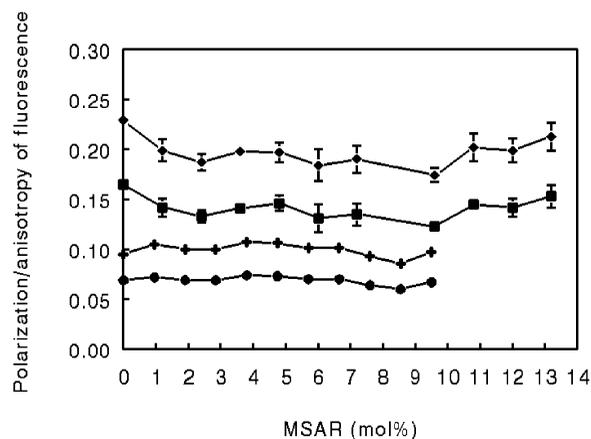


Fig. 3. The effect of MSAR upon fluorescence intensity of incorporated fluorescence dye (TMA-DPH and NBD-PE). (+) NBD-PE fluorescence polarization; (●) NBD-PE fluorescence anisotropy; (◆) TMA-DPH fluorescence polarization; (■) TMA-DPH fluorescence anisotropy.

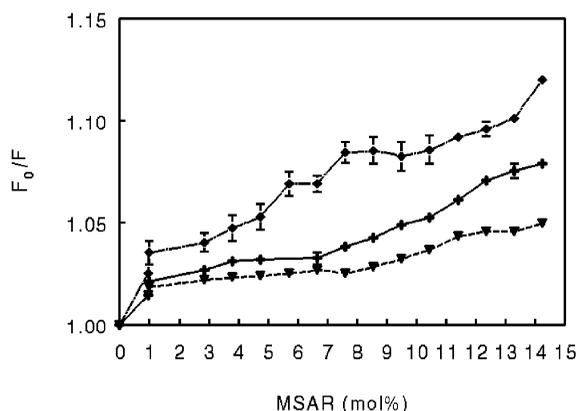


Fig. 4. The effect of MSAR upon fluorescence of 9-anthroxystearic acids. F , fluorescence of sample after addition of MSAR; F_0 , fluorescence of controls (no MSAR added); (+) AS-2; (▼) AS-6; (◆) AS-9. Data are given as means \pm SD ($n = 3-4$).

tion. As the CMC of MSAR was relatively high (0.893 mM), in comparison to the parent pentacyclresorcinol at given concentrations, the number of directly interacting molecules (monomers) with the membrane will be higher. Even if the concentration of MSAR would be above the CMC and micelles formed, they should first dissociate to monomers to keep the equilibrium between these two form. It was demonstrated (Fig. 4) that the 9-AS signal was most intensively quenched by MSAR molecules, suggesting a relatively deep location of these molecules within the lipid bilayer. Molecular and biophysical mechanisms of this process are still unknown.

Effect of MSAR on phase transitions and fluidity of lipid bilayer

In all measurements, both the phase transition temperature (T_c) and the membrane fluidity under and above T_c , of pyrene-labeled phospholipids have been used. In those lipids the chromophore was attached to the distal end of one of the acyl chains [1,2-bis-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine]. This fluorescent dye forms intermolecular excited state dimers within the lipid bilayer. Excited state dimers (excimers) show maximum fluorescence emission shifted to longer wavelengths (red shift) towards their monomeric form. Excimer formation was dependent on the pyrene dye concentration in the phospholipids bilayer and on the molecular lateral diffusion. The I_e/I_m ratio (pyrene excimer/pyrene monomer fluorescence intensity ratio) is a parameter, which could be used to study the lateral organization of phospholipids (*e.g.* microdomain formation), main phase transition temperature (T_c), and the lateral diffusion and membrane fluidity (Somharju, 2002). The latest parameter depends on the temperature and phase state of the bilayer (gel or liquid crystalline state of membrane). Liposomes of DPPC were chosen as our model system because DPPC liposome bilayers are thermodynamically well defined and have been used extensively to examine the physical properties of lipid bilayers under the influence of polymer (Savva *et al.*, 1999; Zhou *et al.*, 2005), peptide (Fresta *et al.*, 2000) and drug or membrane agents (Berquand *et al.*, 2005; Schnitzer *et al.*, 2005) perturbation. Similarly, preparation of liposomes by sonication was used as described in earlier reports (Berquand *et al.*, 2005; Schnitzer *et al.*, 2005; Ishikara *et al.*, 2002;

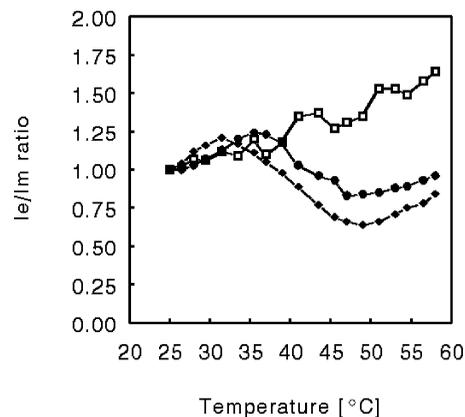


Fig. 5. The effect of MSAR upon forming of excimer/monomer ratio of pyrene-labeled phospholipid. (●) DPPC; (◆) 4 mol% of MSAR; (◻) 10 mol% of MSAR; I_e/I_m , pyrene excimer/pyrene monomer fluorescence intensity ratio.

Pintea *et al.*, 2005). In this experiment molecules of MSAR were incorporated into both leaflets of the phospholipid bilayer and exhibited a concentration-dependent effect on membrane fluidity. Generally, at low contents (1–6 mol% versus total lipid) they cause a significant increase of the phospholipid bilayer fluidity (both under and above the main phase transition temperature) of DPPC liposomes (Fig. 5). The presence of MSAR slightly reduced the T_c value (from 41 °C for only DPPC, 39.5 °C at 5–8 mol% of MSAR to 39 °C at 20 mol% of MSAR as determined by the turbidimetric method) (Table I). Additionally, the presence of MSAR seemed to have a similar effect (cholesterol-like) like the effect of the parent compound that was demonstrated earlier (Kozubek *et al.*, 1988). On the other hand, changes caused in the phospholipid bilayer at a higher content of MSAR (7–10, 15 and 20 mol%) made the determination of the T_c value and the evaluation of the membrane fluidity changes by the application of the pyrene-labeled lipid method impossible (Fig. 5, Table I).

These results suggest possible effects of the studied compound upon packing of the phospholipids within the bilayer by, for instance, the formation of separated phases or other aggregates that affect the behaviour of probe molecules used in the studies. The effects of MSAR on the phospholipase A_2 activity suggest that this compound can cause defects in the phospholipids packing within the bilayer, which is observed as disappearance of

Table I. The values of the main thermotropic phase transition temperatures (T_c) of liposomal phospholipids determined by two different methods.

Content of MSAR (mol%)	Temperature of main phase transition [°C]	
	Turbidimetric method	Pyrene method
0	41.0	41.0
1	40.5	41.0
4	40.0	41.0
7	39.5	(*)
9	40.5	(*)
20	39.0	(*)

(*) Determination of T_c was impossible, see explanations in text.

the first phase of the enzyme kinetic curve (called lag time). In the case of a high level of phase separation in the bilayer it is possible that the thermotropic phase transition could not be observed. In the case of a pyrene method the level of phospholipids packing was measured. Because of that high concentration of MSAR in the liposomal bilayer, determination of T_c was impossible.

Determination of the main phase transition temperature of the MSAR-modified bilayer by the turbidimetric method relied on the analysis of the relationship between light scattering caused by the studied liposomes and the temperature of the sample (Fig. 6). The results did not differ significantly to those estimated by the fluorescence method (Table I). Using the turbidimetric method to investigate

the temperature of the main phase transition of the liposomal bilayer changes of the liposomal shape and size and bilayer relaxation were observed.

Effect of MSAR upon acetylcholinesterase activity

The decrease (inhibition of 2% at 10 μM , 15% at 50 μM , and 40% at 100 μM concentration of MSAR) of the activity of membrane-bound enzyme-acetylcholinesterase, upon incorporation of MSAR may result from the alteration of the distribution and the mobility of membrane phospholipids (fluidity of the bilayer) and/or from the state of enzyme boundary lipids, *i.e.*, lipids that are closely bound/related to the membrane enzyme. In case of acetylcholinesterase, hitherto studied resorcinolic lipids also induced a marked decrease of the apparent activity of this enzyme (Kozubek *et al.*, 1992). The similarity of the net effect of these compounds, noted previously, and the effect of a more polar compound (as merulinic acid or MSAR) may suggest the existence of more complex interactions. These interactions may involve not only the effect of the polar (hydrophilic) region of the bilayer modifier but also the effect of the hydrophobic alk(en)yl side chain. Although MSAR and merulinic acid are similarly negatively charged molecules, they exhibit a different ability to decrease to the apparent acetylcholinesterase activity. Merulinic acid, a molecule that has only one side chain, but a carboxyl ring attached, decreased the acetylcholinesterase activity almost completely at the concentration of 95 μM (Stasiuk *et al.*, 2004) while MSAR, having two side chains, a stronger polar head attached to the ring and similar charge, decreased the acetylcholinesterase activity by only 40% at 100 μM concentration. Alkylresorcinol C15:0 (5-*n*-pentadecylresorcinol), a molecule that has no charged groups (at neutral pH value) exhibited only a moderate effect upon the acetylcholinesterase activity (approx. 20% apparent inhibition at 95 μM). Another charged phenolic lipid – anacardic acid [pentadec(en)ylsalicylic acid], that differs from merulinic acid only by the lack of one hydroxy group, exhibited a similar strong inhibitory effect upon the apparent acetylcholinesterase activity (almost 95% inhibition at 95 μM) (data not shown) suggesting the importance of the charged ring attachments for the observed effect. This effect, however, might be diminished by the presence of the second chain in

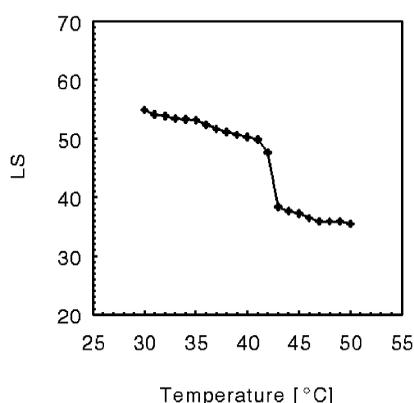


Fig. 6. The effect of MSAR upon main phase transition temperature of the DPPC bilayer determined by the turbidimetric method. LS, light scattering (relative units); (+) 5 mol% of MSAR as an example. Data are given as means \pm SD ($n = 3-4$).

the molecule that, in consequence, may play a significant role in the alteration of the overall amphiphilic properties of the molecule.

The data confirm that both hydrophilic and hydrophobic regions of the amphiphilic biomembrane modulating molecules participate in the alteration of the native enzyme environment by, *e.g.*, inducing the lipid environment-dependent conformational changes of the enzyme molecule and decreasing the spatial adjustment of the enzyme molecule within the actual lipid environment. These changes, in consequence, will lead to the decrease of its apparent activity. Similar effects have been also observed for other amphiphilic molecules (Farias, 1980; Kamber and Kopeikina-Tsiboukidou, 1986).

Effect of MSAR upon phospholipase A₂ activity

The effect of MSAR upon phospholipase A₂ activity was also dependent upon the properties of the lipid bilayer. In general, phospholipase A₂ catalyzes the hydrolysis of the ester bond of the phospholipid at the position *sn*-2. Using pyrene-labeled fatty acids in the phospholipid molecules a decrease of the fluorescence of the sample related to the progress of hydrolysis is observed. The phospholipase A₂ kinetics usually exhibits three stages. During the first stage (lag time) reaction progress is slow and dependent upon the weak binding of the enzyme to the lipid bilayer due to, *e.g.* an insufficient number of lipid packing defects that are necessary for the proper activity of the enzyme (Jain and Berg, 1989; Burack *et al.*, 1993; Jorgensen *et al.*, 2002). The second (burst) and third (steady-state) stages are the consequence of the existence of the appropriate number of packing defects in the bilayer, good binding of the enzyme and its lateral movement required for the efficient hydrolysis of phospholipid molecules. All these phases were visible only in case of DPPC liposomes, not containing MSAR (Fig. 7A). MSAR even at very low membrane content (1 mol%) induced a complete abolishment of the first phase (lag time) (Fig. 7B).

This observation indicates that these molecules induce the formation of packing defects in the bilayer which may result from imperfect mixing of phospholipids and MSAR molecules or increased lipids fluidity that, in consequence, would facilitate the creation of the increasing number of packing defects. The increase of the content of MSAR in

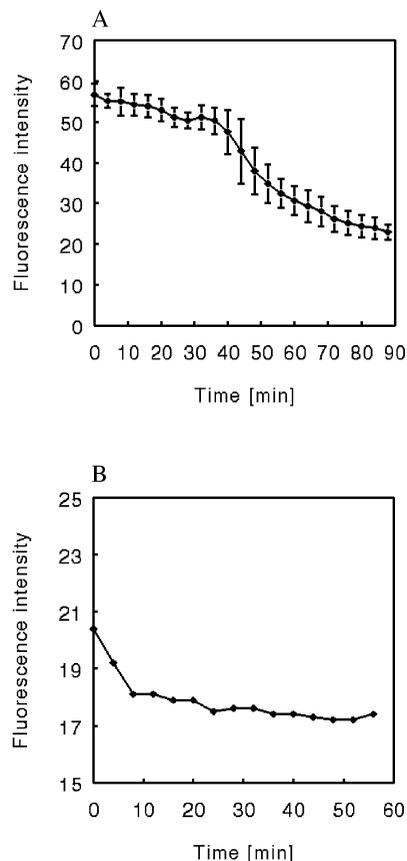


Fig. 7. The effect of MSAR upon phospholipase A₂ activity with respect to (A) DPPC liposomes (control) and (B) DPPC/MSAR (99:1) liposomes. Data are given as means \pm SD ($n = 3-4$).

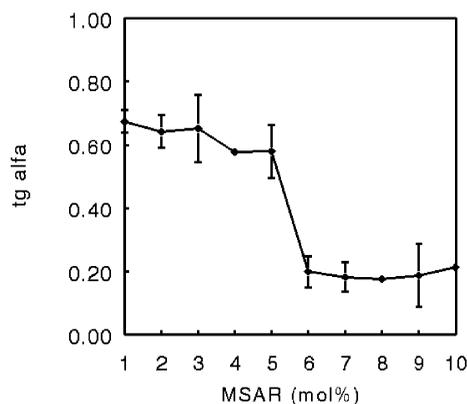


Fig. 8. The effect of MSAR upon phospholipase A₂ activity as illustrated by the values of a tangent of the slope of the kinetic curve of the second stage (burst) PLA₂ reaction. Data are given as means \pm SD ($n = 3-4$).

the bilayer resulted in the increase of the level of hydrolysis at the steady-state phase (Fig. 8). However, above 6 mol% of MSAR, the velocity of the phospholipid hydrolysis in the bilayer was decreased what suggests that above this content MSAR may affect the association of the enzyme with the bilayer as well as the lateral movement of the enzyme which, in consequence, would affect the overall velocity of the phospholipid hydrolysis. This observation is, to some extent, contradictory to the previous data on the effect of 5-*n*-alkylresorcinols upon phospholipase A₂. These compounds, at low bilayer content caused a significant increase of the lag time (Kozubek, 1992). However, the effect of 5-*n*-alkylresorcinols on the second phase was similar to this observed in the study of MSAR. The differences may be explained as

the results of a different effect of these compounds upon lipid bilayer fluidity. The single-chain amphiphile (like 5-*n*-alkylresorcinol) exhibits a rigidifying effect upon the bilayer and protects binding of phospholipase A₂ and its lateral diffusion in the lipid bilayer whereas MSAR, that fluidizes bilayer, will facilitate this process. Both molecules at higher bilayer contents affect the lateral movement of the enzyme, thus decrease the steady-state velocity of the reaction.

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