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Synthesis and characterisation of a new class of stable *S*-adenosyl-L-methionine salts

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Abstract

S-adenosyl-L-methionine (SAM) is an important metabolic intermediate that serves as a donor of methyl and aminopropyl groups to a variety of acceptor molecules. The molecule in vitro is unstable both in solution and in crystalline form undergoing irreversible conversion to 5′-methyltioadenosine (MTA) and homoserine lactone. Since this form of instability seems to be prevented in the cell of the living organism by bonds with macromolecules, we designed and developed a novel class of salts of SAM with large size anions to improve the stability of the sulfonium compound outside the cell. For this purpose we synthesised and characterised by NMR and IR spectroscopy anions consisting of amidic derivatives of taurine with fatty acids. Stability studies performed with the new SAM salts indicate that SAM becomes much more stable when it interacts with large size anions and in fact, more than 84% of the SAM is recovered after 36 months in lyophilized samples. The high stability of the new products widens the possibility of new therapeutic applications of SAM in human therapy.

Keywords: *S*-adenosyl-L-methionine; Sodium *N*-ole-1-oyltaurate; Stability; Liposoluble derivatives

1. Introduction

S-Adenosyl-L-methionine (SAM), the only natural sulfonium compound present in mammals, is involved in many crucial biochemical processes. Undoubtedly, the most investigated SAM-dependent reactions are the enzymatic transmethylations, in which a methyl group is transferred from SAM to a wide variety of methyl acceptors, the biosynthesis of aliphatic polyamine and the transsulphuration reactions (Paik et al., 1975; Giulidori et al., 1984; Prased et al., 1985).

The well-established biochemical roles and pharmacological effects of the sulfonium compound justify the great interest in its therapeutic use (Friedel et al., 1989). SAM prevents the reduction of phospholipid methylation, thus maintaining membrane fluidity, it protects the liver against

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lesive agents and it is administered in human therapy in depressive syndromes and in osteoarthritis (Cimino et al., 1984; Barcelo et al., 1990; Osman et al., 1993; Varela-Moreiras et al., 1995).

Moreover, the main problem of this molecule is represented by its extreme instability at ambient or above ambient temperatures, at neutral and alkaline pH and in presence of humidity since a very little amount of water is required to start the degradation reactions.

Degradation occurs by different mechanisms, the most important of which leads to the formation of 5′-methyltioadenosine (MTA) and homoserine lactone through a nucleophilic intramolecular attack by the oxygen of the carboxylate group on the γ-carbon atom of the aminoacidic chain. The other degradation reactions are the conversion to the biologically inactive (*R*,*S*)-SAM stereoisomer (being *S*,*S* — the configuration of the active molecule) and the hydrolysis to adenine and *S*-pentosylmethionine (Borchardt, 1979; Hoffman, 1986). For this reason, research about SAM stabilisation has been directed towards the preparation of salts which are stable under normal temperature and humidity conditions. SAM sulfate and chloride have been produced but they can be used only as reagents in biochemistry for short times, because even in the dry state the stability is limited in time at low temperatures.

On the bases of the hypothesis that in the cell SAM instability can be prevented through binding to macromolecules, SAM salts with large anions were produced (Smith, 1976). The most stable SAM salts so far known are the double salts with sulfate and *p*-toluenesulfonate anions patented by Fiecchi and at present administered in therapy (Fiecchi, 1976). In general, SAM stability is correlated to the anion size since by increasing the steric hindrance of the anions SAM stability is improved (Barbato et al., 1989).

In agreement with this observation and in order to further improve SAM stability over time, we prepared new stable SAM salts with large size anions constituted by amidic derivatives of taurine with organic acids having a number of carbon atoms ranging from 8 to 18.

The present paper reports the results of stability tests for the derivative SAM-*N*-ole-1-oyltaurate, both for anhydrous and liposome preparations. The stability investigation was limited to SAM-*N*-ole-1-oyltaurate salt since the synthesis of this anion and its precipitation with SAM gave better yield.

2. Materials

Caprylic acid, myristic acid, stearic acid, oleic acid, taurine and 1,1′-carbonyldiimidazole were obtained from Sigma (St. Louis, MO, USA). Precoated plates of silica gel 60 F_{254} were purchased from Merck (Darmstadt, Germany). SAM sulfate was prepared from L-methionine-enriched yeast grown in our laboratory. DMSO-d_6, CD_3OD and $CDCl_3$ solvents for magnetic resonance analysis were purchased from Aldrich Chimica (Milan, Italy).

3. Methods

3.1. Synthesis of amidic derivatives of taurine

Amidic derivatives were synthesised according to the following procedure. The carboxylic function was activated by a molar excess of 1,1′-carbonyldiimidazole (CDI) in the absence of solvents at variable temperatures according to the acid used. CDI was added in small aliquots and the conversion of the acid into the corresponding imidazolide was complete when no more CO_2 from the reaction mixture was generated.

The second step of the synthesis consisted of the reaction between imidazolide and taurine. A suspension of taurine in *N*,*N*-dimethylformamide (DMF) was added to the reaction mixture and stirred vigorously for 24 h at different temperatures in relation to the different imidazolide used. At the end of the reaction DMF was distilled and the resulting mixture was treated with diethylic ether to remove the residual organic acid.

The synthesised products were solubilized in water and further purified by dialysis, since they formed micellar aggregates which did not cross

Table 1
Reagents and temperatures utilized in the syntheses of the sodium acyloyltaurate salts[a]

Acid (mmol)	CDI (mmol)	Taurine (mmol)	DMF (ml)	Temperature (°C)	Yield (%)
Caprylic 69.3	104	69.3	43.3	70	A, 50
Myristic 48	72	48	30	70	B, 80
Stearic 35	52.5	35	21.9	80	C, 80
Oleic 35.5	53.25	35.5	22.2	60	D, 88

[a] CDI, 1,1′-carbonyldiimidazole; DMF, *N*,*N*-dimethylformamide. A, sodium *N*-capryl-1-oyltaurate $CH_3(CH_2)_6CONHCH_2CH_2SO_3^-Na^+$; B, sodium *N*-myrist-1-oyltaurate $CH_3(CH_2)_{12}CONHCH_2CH_2SO_3^-Na^+$; C, sodium *N*-stear-1-oyltaurate $CH_3(CH_2)_{16}CONHCH_2CH_2SO_3^-Na^+$; D, sodium *N*-ole-1-oyltaurate $CH_3(CH_2)_7CH=CH(CH_2)_7CONHCH_2CH_2SO_3^-Na^+$.

the dialysis membrane (cut-off 10–14 kDa), whereas taurine, imidazole, and CDI were easily removed. To convert the imidazole acyloyltaurate salts into sodium salts, the products were dialyzed versus 1 M NaCl and, then, versus distilled water. The compounds obtained were frozen and lyophilized. The temperatures and the amount of reagents used in each synthesis are reported in Table 1.

The time course of the reactions was followed by thin layer chromatography. Pre-coated plates of silica gel 60 F_{254} were developed with a mixture of *n*-butanol/acetic acid/water (65/25/15 by vol.) and the reaction products were revealed by using ultraviolet light.

In order to assess the formation of the amides infra-red, ^1H and ^{13}C NMR spectra of the synthesised sodium salts were recorded by using an FT infra-red instrument and a 200 MHz Bruker instrument. In Table 2 are reported the characteristic infra-red values of stretching of the carbonyl group and the ^1H and ^{13}C chemical shifts of the synthesised amidic bonds.

3.2. Preparation of SAM sulfate

SAM sulfate was prepared microbiologically using L-methionine-enriched *Saccharomyces cerevisiae*. The culture medium was prepared according to Schlenk with the following modifications: yeast extract (0.025 g/l), L-methionine (1.5 g/l) and commercial bakers' yeast (30 g/l) (Schlenk et al., 1965). The growth was performed at 37°C in a Dubnoff incubator under energetic stirrer. Yeast cells were harvested after 18 h and treated with 1/10 vol. of ethyl acetate. SAM was extracted by washing the permeabilized cells with 1 vol. of 0.5 M sulfuric acid and cell debris was removed by centrifugation in a Beckman centrifuge at 12 100 × *g* in a JA-20 rotor at 4°C for 30 min. The clear supernatant (pH 5.0) was loaded onto a column of Amberlite IRC-50 and elution was started with 1 vol. of 0.5% (v/v) acetic acid to eliminate the undesired nucleotide fragments. SAM was subsequently eluted with 25 mM sulfuric acid and its purity was checked by a Beckman System Gold liquid chromatograph equipped with

Table 2
Characteristic absorptions of the amidic bonds of the synthesised sodium salts[a]

Compound	IR, stretching **CO**NH (cm^{-1})	^1H NMR, chemical shift CONH ppm	^{13}C NMR, chemical shift **C**ONH ppm
A	1630	7.70 (1)	176.23 (1)
B	1650	7.70 (1)	176.20 (2)
C	1650	7.68 (1)	174.01 (3)
D	1650	7.70 (1)	172.63 (2)

[a] A, sodium *N*-capryl-1-oyltaurate; B, sodium *N*-myrist-1-oyltaurate; C, sodium *N*-stear-1-oyltaurate; D, sodium *N*-ole-1-oyltaurate. NMR spectra were recorded using tetramethylsylane (TMS) as internal standard and the following deuterated solvents: (1) DMSO-d_6; (2) CD_3OD; (3) CD_3OD:$CDCl_3$ 1/1 (v/v).

an Ultrasil column (10-μm particle size, 250 × 4.6 mm ID) and an ultraviolet detector operating at 254 nm. Elution was carried out with 0.4 M ammonium formate buffer (pH 4.0) at a flow rate of 1 ml/min.

3.3. Preparation of liposoluble SAM salts

Liposoluble derivatives of SAM with the synthesised sodium salts were prepared by precipitating a solution of SAM sulfate with different amounts of sodium acyloyltaurate at low pH. The SAM-acyloyltaurate molar ratio of the salts obtained ranged from 1:4 to 1:6.

According to a standard procedure, the SAM-*N*-ole-1-oyltaurate salt was prepared as follows: 4.41 g of sodium *N*-ole-1-oyltaurate (10.72 mmol) were solubilized in 53.6 ml of distilled water (final pH 1.0 with 2 M H_2SO_4). This solution was slowly added, under vigorous stirring, to 100 mM SAM sulfate (26.8 ml, 2.68 mmol) and the solution immediately became muddy giving a white precipitate. The mixture was centrifuged in a Beckman centrifuge at 12 100 × *g* for 45 min at 4°C and the resulting precipitate was recovered and lyophilized. After grinding, a fine, clear-coloured, low hygroscopic powder, with 1:4 SAM-*N*-ole-1-oyltaurate molar ratio, was obtained. The SAM precipitation yield was 75%. The same procedure was followed for the preparation of other SAM salts.

3.4. Characterisation of liposoluble SAM salts

The SAM-acyloyltaurate molar ratio of the salts prepared was determined by HPLC in the conditions reported above. A total of 5 mg of the lyophilized products was solubilized in 5 ml of 0.4 M ammonium formate buffer (pH 7.0) and aliquots of 10 μl were analyzed. The SAM amount in the liposoluble salts was calculated using a calibration curve ranging from 2.5 to 10 nmol. By assessing the weight difference between the lyophilized samples and their SAM content it was possible to determine the SAM-acyloyltaurate molar ratio.

3.5. Preparation of liposomes

Liposomes of SAM-*N*-ole-1-oyltaurate (1:4 molar ratio) were prepared by sonificating SAM-*N*-ole-1-oyltaurate (SAM 1.5 mg/ml) in an ice bath for 20 min in 0.1 M citrate-phosphate buffer, pH 2.5, and in 0.4 M ammonium formate buffer, pH 4.0 and 7.0. (Sonicator Heat System).

3.6. Stability evaluation

The following samples were analyzed: (1) lyophilized SAM-*N*-ole-1-oyltaurate salts with 1:4, 1:5 and 1:6 molar ratio stored at room temperature in (a) unsealed glass vials; (b) unsealed glass vials in the presence of P_2O_5; (c) vacuum-sealed glass vials; and (2) liposomes of SAM-*N*-ole-1-oyltaurate (1:4 molar ratio) stored at room temperature.

SAM stability was monitored by HPLC under the conditions described above.

4. Results and discussion

The development of a novel class of stable and unhygroscopic SAM salts was approached by synthesising anions with high steric hindrance constituted by amides of taurine and fatty acids with carbon chains ranging from 8 to 18 atoms. The SAM salts were then easily prepared by precipitating the sulfonium compound with an acidic solution of the synthesised anions.

The SAM was precipitated in high yield (90–97%) by sodium acyloyltaurate with carbon chains of 14 or more atoms, whereas no insoluble salts were obtained with saturated or unsaturated short chains, such as sodium *N*-capryl-1-oyltaurate. These findings underline the importance of anion size in determining the precipitation of SAM.

However not only the size but also the amount of anion played a role in the yield of SAM precipitation. As shown in Fig. 1, when the SAM-*N*-ole-1-oyltaurate molar ratio was 1:1, less than 20% of SAM precipitated. By increasing the concentration of the anion, the amount of SAM

precipitated increased obtaining almost 100% precipitation when using 1:6 molar ratio. In all cases the molar ratio of the recovered SAM salts started from 1:4, indicating that it was not possible to obtain salts with low molar ratio since a critical quantity of anions was necessary to precipitate SAM.

The general formula of the final products is: SAM_n (R-CO-NH-$(CH_2)_2SO_3^-)_m$, where R-CO is the acylic residue of the acid used, n varies from 0 to 2 and m from 4 to 6. The total number of SAM charges is strictly pH-dependent. The molecule has three ionizable groups besides the sulfonium pole: the carboxyl and the aminic groups of the aminoacidic chain with pK_a 1.8 and 7.8, respectively and the aminic group of the purine with pK_a 3.4 (Farooqui et al., 1983). Consequently, at low pH SAM has three positive charges and the interactions with the anions are of electrostatic origin. When the anion:SAM molar ratio is higher than three not all the anions can be neutralized by the positive charges and the hydrophobic moieties interact among them forming micellar aggregates which lead to the precipitation of SAM.

The synthesised SAM salts are soluble at physiological pH since in neutral and alkaline solutions the positive charges decrease with reduction of number of the interacting anions and liberation of the sulfonium compound.

The SAM salts are also soluble in chloroform up to 10% (w/v), thus confirming their structural organization in form of micelles with the hydrophobic chains directed towards the external environment and the polar heads, surrounding the SAM, inside.

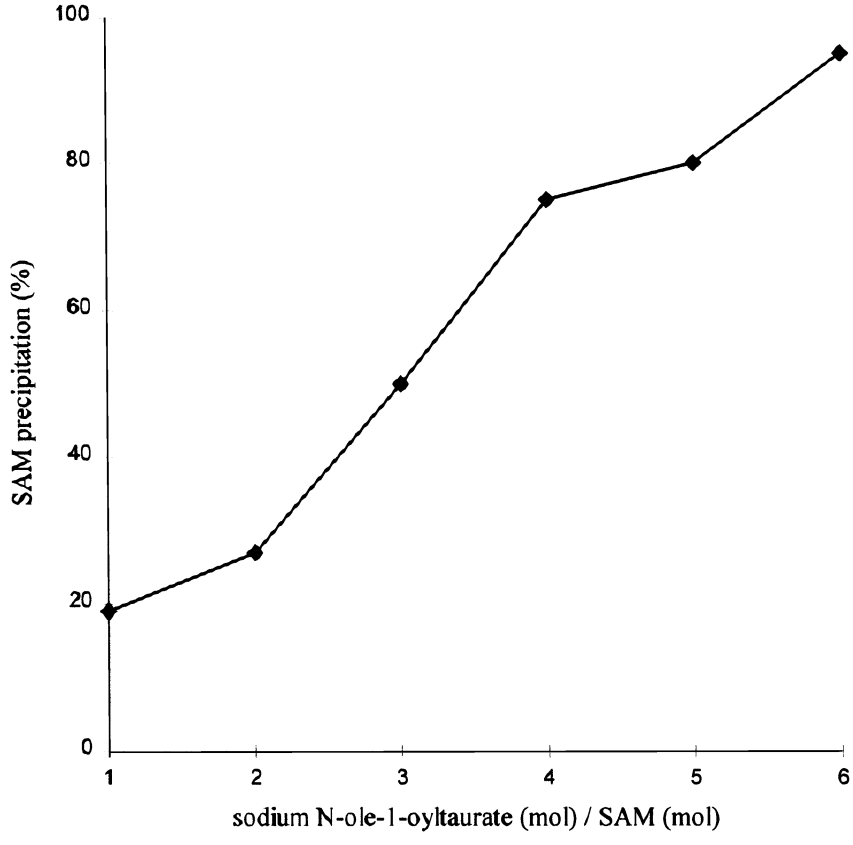

Fig. 1. Yield of precipitated SAM with increasing amounts of sodium *N*-ole-1-oyltaurate.

Table 3
Stability data of SAM-*N*-ole-1-oyltaurate lyophilized samples[a]

Molar ratio (SAM:*N*-ole-1-oyltaurate)	SAM (%)			
	18 months		36 months	
	A	B	A	B
1:4	100	100	89	92
1:5	100	100	84	91
1:6	100	100	93	93

[a] Data refer to SAM-*N*-ole-1-oyltaurate lyophilized samples stored at room temperature in glass vials in the presence of P_2O_5 (A) or under vacuum (B). SAM content was determined after 18 and 36 months by HPLC as described in Section 3.

The results of stability tests of the lyophylized SAM-*N*-ole-1-oyltaurate salts with 1:4, 1:5 and 1:6 molar ratio kept at room temperature in unsealed glass vials demonstrated that over 18 months $\sim$10% of SAM was lost, whereas in double salts with sulfate and *p*-toluensulfonate anions, used as control, the loss was 12% in 1 month, indicating the important role of the *N*-ole-1-oyltaurate in protecting the SAM. This role can be explained in terms of interactions with the SAM, since the anions which form the micelles cause a conformational stiffening of the aminoacidic chain of the sulfonium molecule preventing the intramolecular degradation reaction.

The SAM salts stability in lyophilized samples stored in the presence of P_2O_5 and under vacuum is reported in Table 3. In these conditions no SAM degradation occurred over 18 months and this was attributed to the dehydrated environment created by the storage methods. Even after 36 months the recovery of SAM was high; it was maximal (93%) in samples with 1:6 molar ratio and was due to the larger quantity of *N*-ole-1-oyltaurate and consequently to a greater protective effect of this anion.

In order to establish the rate of SAM degradation in function of pH, SAM-*N*-ole-1-oyltaurate liposomes (1:4 molar ratio) at pH 2.5, 4.0 and 7.0 were prepared and analyzed over time (Fig. 2A). SAM sulfate solutions were used as control (Fig. 2B). As expected, the SAM degradation rate was directly proportional to pH. In fact the sulfonium compound decreased more rapidly at pH 7.0 both in the SAM-*N*-ole-1-oyltaurate preparation and in the SAM sulfate solution because at neutral pH the anions which protect SAM from the nucleophilic attack responsible for MTA formation decrease, exposing the sulfonium compound to the degradative reaction.

In all liposome preparations the SAM degradation was slower with respect to the controls. After 3 months, SAM was totally degraded in the control at pH 7.0, while more than double this time was required for its complete disappearance in the presence of *N*-ole-1-oyltaurate. Moreover, after 8 months 8 and 26% of SAM was still measured in SAM-*N*-ole-1-oyltaurate samples at pH 4.0 and 2.5, respectively, while no traces of the molecule were detected in the controls after a shorter time.

5. Conclusions

Lyophilized SAM-*N*-ole-1-oyltaurate salts are highly stable. In addition the low hygroscopicity of the samples, which is due to the strong hydrophobicity of the anion, as well as the conditions preventing contact with humidity, such as in vacuo, or the presence of dehydrating agents like P_2O_5, permit their storage over long time.

The stability investigation was also extended to liposome preparations in order to study the behaviour of the sulfonium compound in function of the pH. The results indicate that SAM stability was improved by the presence of a large anion such as *N*-ole-1-oyltaurate with respect to the samples of SAM sulfate at any pH values.

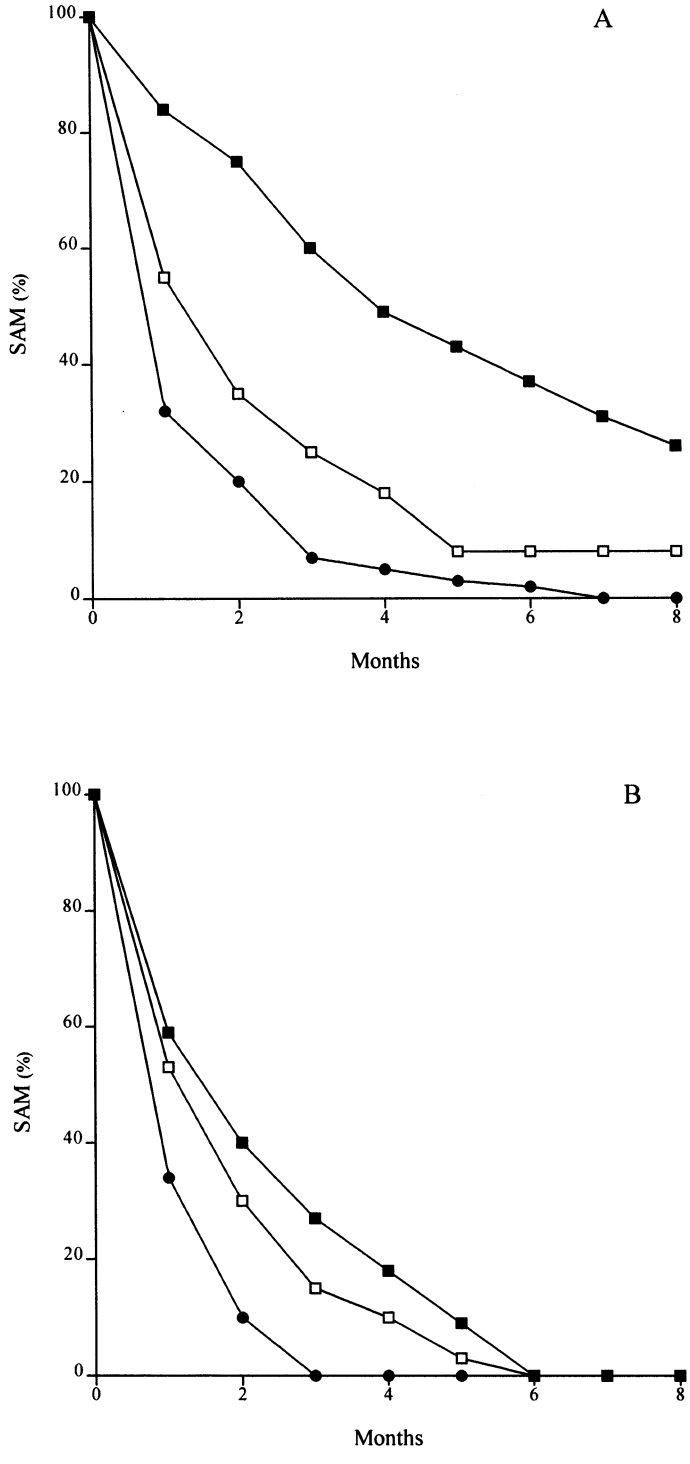

Fig. 2. Stability of liposome preparations of SAM-*N*-ole-1-oyltaurate (1:4 molar ratio) (A) and SAM sulfate solutions (B) prepared at different pH and stored at room temperature (■, pH 2.5; □, pH 4.0; ●, pH 7.0). The samples were analyzed at each time point by HPLC as described in Section 3.

The high stability shown by the SAM salts described could be a valid alternative to the use of hygroscopic SAM sulfate and *p*-toluenesulfonate compounds at present used in therapy both as parenteral and oral formulations. In addition, another important aspect of the novel products to underline is represented by their liposolubility. Consequently, they may have an improved bioavailability compared to the salts currently administered, thus allowing a more advantageous utilization of SAM in therapy. The liposolubility exhibited by the new SAM salts makes these products particularly suitable for oral administration since an easy absorption process by the cell membrane is a function of the liposolubility of the active principle.

In order to follow the metabolic fate of SAM when it is orally administered as SAM-*N*-ole-1-oyltaurate salt in rats, a pharmacokinetic study is currently in progress in our laboratory.

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