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Characterization of the macrocyclic carbon suboxide factors as potent Na,K-ATPase and SR Ca-ATPase inhibitors

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Abstract

Recently discovered macrocyclic carbon suboxide (MCS) factors with the general formula $(C_3O_2)_n$ were found to strongly inhibit rabbit and rat Na,K-ATPase as well as SR Ca-ATPase. Highly active MCS factors were obtained by a base/acid treatment of their lipophilic precursor isolated from plants. In the ESI-MS spectra, the dominant molar mass ion of 431 Da corresponds to a 1:1 complex of the carbon suboxide hexamer ($n=6$; $M_r=408$ Da) with a Na^+ ion. Additional mass ions identified in positive and negative ion mode were assigned as complexes of the MCS hexamer ($n=6$) and octamer ($n=8$) with Na^+ or with TFA^- in various ratios. The dominant mass ion values of these active MCS factors from plants are also found in mass spectra of previously described endogenous digitalis-like factors (EDLF) from animals. This would suggest that ubiquitously distributed MCS factors may function as putative endogenous regulatory substances of Na,K-ATPase and possibly of other ATPases. With the symmetric display of several equivalent carbonyl or hydroxy groups, the structure of MCS factors is particularly suited for interactions with proteins and other bio-molecules. This could explain the high biological activity and the unusual properties of the MCS factors.

Keywords: Macrocyclic carbon suboxide; Na,K-ATPase; Ca-ATPase; Endogenous regulatory factor; Enzymatic activity

1. Introduction

The Na,K-ATPase (EC 3.6.1.37) or sodium pump is a plasma membrane protein ubiquitously present in all higher eucaryotic cells. It belongs to the larger family of P-type ATPases with the common feature of forming a phosphorylated intermediate during the pump cycle. Sodium pumps control a broad spectrum of essential cellular functions such as ionic homeostasis, membrane potential, pH, temperature, water osmosis [1], thereby regulating different physiological processes, e.g. muscle contraction, nervous signal transmission, renal sodium retention, vascular tone, etc. [2]. This multiple regulatory potential of the same enzyme is explained by the existence of tissue-specific assemblies of different structural subunits or isoforms [3]. The physiological role of the various isoforms may consist of different sensitivities towards endogenous regulators of the pump [4]. Malfunctions of the Na,K-ATPase are clearly involved in several pathologies such as essential hypertension, cardiac failure, diabetes and others [5,6]. It is therefore of major therapeutic interest to elucidate the physiological regulatory mechanism of the Na,K-ATPase which is—despite all efforts—still not well understood.

Calcium homeostasis inside the eucaryotic cells is maintained by the ubiquitously distributed membrane enzymes known as Ca-ATPases or Ca pumps. The plasma membrane Ca-ATPase generally counteracts the influx of free Ca^{2+} ions through calcium channels and thus exerts an essential role in controlling enzymatic reactions and a broad spectrum of intracellular signaling processes [7]. In muscle cells, the SR Ca-ATPase pumps Ca^{2+} ions back into the sarcoplasmic reticulum stores during muscle relaxation. Ca-ATPase is structurally related to the Na,K-ATPase [8], both belonging to the super-family of P-type ATPases evolved presumably from a common archaic ancestor 3.5 billion years ago [9].

Extensive studies performed in the past support the presence in animal tissues and fluids of some endogenous inhibitors of the sodium pump (called endogenous digitalis-

Abbreviations: MCS, macrocyclic carbon suboxide; SR, sarcoplasmic reticulum; TFA, trifluoracetic acid

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like factors, EDLFs) [10–12]. However, their chemical nature remained elusive, and digitalis steroids and particularly ouabain are still discussed as the putative endogenous ligand candidates [13,14]. It is, however, rather improbable that digitalis steroids exert this general function, since they are: highly toxic for animals, biosynthesized only by a few rare plants and toads, inactive towards some Na,K-ATPase isoforms, and generally towards Ca-ATPase and other P-type ATPases. So far, all evidences confirm the original statement of Szent-Györgyi [15] that cardiac steroids are only some substitutes of the actual endogenous regulatory factors and nothing more.

Recently, a new class of natural products defined as macrocyclic carbon suboxide (MCS) factors with the general formula $(C_3O_2)_n$ was identified as potent sodium pump inhibitors [16,17]. The proposed structure of these MCS factors corresponds to head-to-tail condensed pyran-4-one rings additionally joined in a cylindrical macrocycle as shown in Fig. 1 for $n=6$ and $n=8$.

The repeating unit of this macrocyclic structural frame is carbon suboxide O=C=C=C=O, the known homologue of CO and CO_2 [18]. Thus, the macrocyclic compounds reported in Fig. 1 are actually the cyclohexamer $(C_3O_2)_6$ and cyclooctamer $(C_3O_2)_8$ of the inorganic carbon suboxide: C_3O_2. This structural assumption is in full agreement with the experimentally determined molar masses of 408.2 and 544.2 Da for the cyclohexamer $(C_3O_2)_6$ and cyclooctamer $(C_3O_2)_8$, respectively. These cyclo-oligomers of the carbon suboxide may be classified both as inorganic and organic substances.

Carbon suboxide may be generated from carbon monoxide by simple electric discharges; thus the presence of C_3O_2 in the archaic earth atmosphere, rich in CO, is very probable. In this sense, MCS factors could be present in the earlier phase of the evolution when the ancestor P-type ATPases evolved [9,17].

The MCS factors were isolated from herbal sources in the form of a structurally less defined lipophilic precursor [16]. In the present communication, a simple and convenient method is described to convert this lipophilic MCS precursor into a hydrophilic form with significantly enhanced inhibitory potency. HPLC and mass spectrometry of the active MCS factors served to characterize these active forms. Their inhibitory activity on the sodium pump from rabbit and rat was compared to that of ouabain. The similarly potent inhibition of SR Ca-ATPase by the MCS factors is additionally reported.

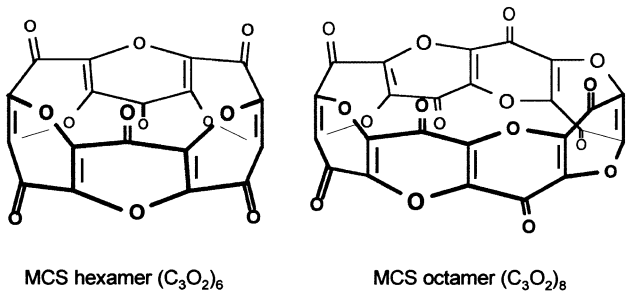

Fig. 1. Structure of the MCS hexamer and octamer.

2. Materials and methods

Phosphoenolpyruvate (PEP), pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt) were from Boehringer (Mannheim). All other reagents were purchased from Merck (Darmstadt) or Sigma-Aldrich (Deisenhofen) at the highest quality available.

Analytical HPLC was carried out on Kontron-Instruments device using a 250/4 mm Nucleosil RP 100-5 column (Macherey-Nagel, Düren, Germany) with a linear gradient of 5–95% acetonitrile in water (both containing 0.05% TFA) for 45 min and at a flow rate of 1.0 ml/min and detection with DAD-440. For LC-MS the microgradient system type 140c (Perkin-Elmer) coupled to PE SCIEX API 165 ESI detector was used with a Nucleosil 2/100 column and a linear gradient of 5–95% acetonitrile in water (0.05% TFA) in 20 min. The ESI mass spectra were recorded in both the positive and negative ion mode.

2.1. Isolation of the lipophilic MCS precursor

Hexane-defatted roots (1.0 kg) of *Helleborus purpurascens* were stirred for 24 h at 20 °C in 70% EtOH (8.0 l), and the resulting solution was concentrated under reduced pressure to a volume of 1.5 l. The aqueous emulsion was then extracted twice with a mixture of hexane (1.2 l)/chloroform (0.3 l), the aqueous phase was separated and acidified to pH 1.5 and extracted twice with *tert*-butyl methyl ether (1.2 l). The organic phase was washed to neutral pH with 0.2 M NaCl, dried over Na_2SO_4 and evaporated to dryness. The resulting brownish, crude lipophilic product (yield: 1.8–3.5 g) was purified roughly by solving it in acetone (50 mg/ml) and mixing the acetone solution with 10-fold volume of *n*-hexane. The vacuum-dried precipitate was further purified by semi-preparative HPLC on a SP 250/10 mm Nucleosil 100-5 C-18 HD column (Macherey-Nagel) at a flow rate of 3.0 ml/min with a linear gradient of 5–90% acetonitrile/water (both containing 0.1% TFA) for 45 min. This chromatographic purification was repeated three to five times until the broad symmetrical HPLC peak of the lipophilic product was free of any impurity detectable spectroscopically in HPLC (at 205 nm) and of organic impurities detectable by TLC (silica plates, Merck) by visualizing with the anisaldehyde/sulfuric acid reagent. The absence, even in trace amounts, of so far isolated organic compounds from *Helleborus* species extracts, e.g. steroid glycosides, saponines, ecdysones, lactones, etc., was verified separately by mass spectrometry. Final yield of the highly purified lipophilic precursor: 27–32 mg calculated for 1 kg dried *Helleborus* roots.

2.2. Preparation of the active MCS factors

The purified and lyophilized lipophilic precursor (1 mg) was dissolved in 1 ml 50% EtOH, diluted with 1.2 M NaOH (5 ml) to a final concentration of 0.167 mg/ml. The alkaline solution was boiled in a round-bottom flask equipped with a water-cooled condenser for 12 h under slow stirring in an oil bath (116 °C). The optimal boiling time for activation of the lipophilic component was found to be 8–14 h (vide infra). After boiling, the alkaline MCS solution was adjusted with 1 M HCl first to a pH value of 8.0–9.6 and then to a final pH of 3 or less. To remove the excess Na^+ ions after boiling in NaOH, the acidified solution was treated with a strong cation-exchanger resin, e.g. for 150 μl MCS-402 solution (pH 3) 40 mg of Amberlyst 15 were used resulting the probe: MCS-L04. The activity of the Na-free MCS solutions is well preserved by storage at +4 °C for 6 months and longer. Several details in the preparation of the highly active MCS factors are apparently of decisive importance, although not fully understood. Thus, a too rapid acidification of the boiled alkaline probe from pH >13 to acidic pH values can lead to a gel-like inactive substance. Titration with diluted HCl (less than 1 M) produced very potent inhibitors, but their activity decayed within a few days' storage at room temperature.

2.3. Enzyme assays

Membrane preparations with a high concentration of Na,K-ATPase (about 5000 pumps per μm^2) were prepared from the outer medulla of rabbit and rat kidneys using procedure C of Jørgensen [19]. The specific ATPase activity was in the range of 2000–2400 μmol P_i/h/mg protein at 37 °C for the rabbit enzyme and 2000 μmol P_i/h/mg protein for the rat enzyme. The enzyme activity of the Na,K-ATPase [22] was determined in a buffer containing 25 mM imidazole (pH 7.2), 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1.5 mM Na_2ATP, 5 nM Na,K-ATPase, 2 mM PEP, 450 units/ml of pyruvate kinase and lactate dehydrogenase, and initially 80 μM NADH. All experiments were performed at 37 °C. The enzyme activity in absence of inhibitor was used as reference. Molar concentration of the MCS solutions was calculated on the basis of a M_r of 408.2 Da because the actual ratio between MCS cyclohexamer and cyclooctamer could not be established precisely. Using the lower M_r instead of the 544.2 Da for the octamer resulted in a more conservative estimate with possibly higher molar concentration of the inhibitor and, therefore, lower values of the specific activity. The pyruvate kinase/lactate dehydrogenase assay was not affected by MCS. The inhibitory action of the MCS compounds was fully developed within the time of mixing the buffer in the cuvette with the inhibitor solution added (1–10 μl). In the presence of excess ouabain, the Na,K-ATPase preparation was fully inhibited, a fact which confirmed the high degree of purity of the enzyme preparation as additionally controlled by SDS electrophoresis (data not shown). The normalized specific activity was calculated as the ratio of the residual activity upon addition of increasing amounts of MCS, and the reference activity.

Ca-ATPase was prepared from rabbit psoas muscle by a slight modification of the method of Heilmann et al. [20]. The whole procedure was performed at temperatures below 4 °C. The protein content of the membrane preparation was determined as described previously [21] and was found to be 2–3 mg/ml for the most active fractions after the final density gradient separation. The specific enzymatic activity was about 2 μmol P_i/h/mg protein at 20 °C. The enzyme activity was determined by the same coupled pyruvate kinase/lactate dehydrogenase assay as in the case of the Na,K-ATPase using buffer (pH 7.5) containing 25 mM HEPES, 1 mM $MgCl_2$, 50 mM KCl, and 0.2 mM Ca^{2+}. Background enzyme activity of the isolated preparation was obtained by addition of 1 μM tharpsigargin. The specific activity of the Ca-ATPase preparation was ~1.8 units/mg at 20 °C and pH 7.5 (which corresponds to 1.8 μmol ATP hydrolyzed per mg protein per min).

3. Results and discussion

3.1. HPLC characterization of the MCS factors

In the analytical HPLC analysis the purified lipophilic precursor elutes as a broad symmetric peak at $t_R = 24.6$ min which corresponds to ~54% acetonitrile concentration (Fig. 2). Standard cardiac glycosides elute under identical conditions at $t_R = 13.3$ min (ouabain), 19.1 min (hellebrin) and 22.2 min (digoxin). The pure active MCS factors resulting from the base/acid treatment elute shortly after the injection peak as shown in a HPLC diagram of a mixture of the active substance with its lipophilic precursor (Fig. 2). However, MCS factors show a pronounced tendency to co-elute with other substances. This was proved by HPLC-MS runs with selected mass ion (SMI) detection, i.e. of 409, 431 or 521 Da (vide infra).

Broad HPLC elution is explained by the existence of self-associations between variously structured MCS factors, especially through semi-acetal bonds between carbonyl and hydroxy groups. HPLC co-elution with other substances are similarly assigned to possibly strong associations between MCS factors and carbohydrates, peptides and similar substances. Both kind of associations are particularly enhanced by the symmetric display of several equivalent carbonyl or hydroxy groups in the structure of the MCS factors (Figs. 1 and 5).

3.2. Mass spectrometric analysis of the MCS factors

In Fig. 3, the ESI-MS spectrum of the highly active preparation: MCS-L04 recorded in the positive-ion mode is reported. The principal mass ion peaks (m/z) correspond to complexes between the MCS hexamer $(C_3O_2)_6$ or octamer

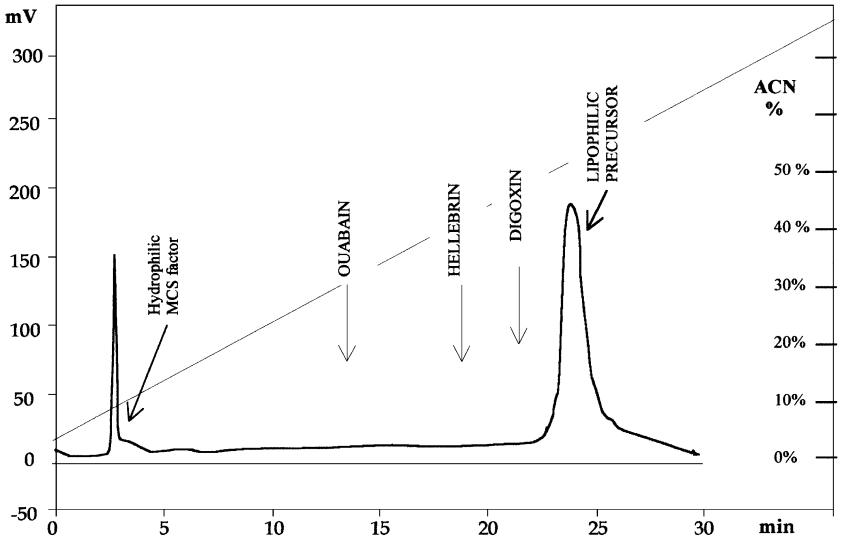

Fig. 2. HPLC elution of the lipophilic precursor and of the highly active hydrophilic MCS factor prepared by the base/acid treatment, compared with standard cardiac steroids.

$(C_3O_2)_8$ and Na^+. The mass ion peak at 431.2 Da was therefore assigned to a 1:1 complex of the MCS hexamer with Na^+, while the peaks with m/z = 295.2 and 159.0 Da correspond to the $[2(C_3O_2)_6 + 3Na^+]$ and $[(C_3O_2)_6 + 3Na^+]$ complexes. These multiple-charge mass ion complexes are generated presumably in the ionization chamber from the more stable 1:1 complex in a mode similar to that reported for the crown–ether complexes with alkali ions [23]. The positive mass ion peak at 567.2 Da was assigned to a 1:1 complex of the 544 Da octamer $(C_3O_2)_8$ with Na^+. Assuming the existence of multiple charged mass ions, the m/z = 295.2 Da (see above) may also be assigned to the complex $[(C_3O_2)_8 + 2Na^+]$, while the m/z = 159 Da peak may be the $[(C_3O_2)_8 + 4Na^+]$ complex. In the positive mode spectrum additional mass ion peaks of weak intensity were detected at 353.0, 489.0, and 624.8 Da which should correspond to complexes between one Na^+ ion and MCS forms with $n = 5$, 7 and 9. Since the MCS oligomers as odd multiples of carbon suboxide are less probable, these may be generated in situ in the ionization chamber.

Interestingly, the principal mass ion values of the MCS factors in the positive ion mode can also be found in the mass spectra of EDLF isolated from animal tissues and fluids. In the FAB-MS spectrum of the EDLF isolated from human plasma [24], the 409 Da peak was considered as the molar mass ion, but without any structural proposal. The further mass ion peaks present at 431 and at 447 Da were assigned as adducts of Na^+ and K^+ to the molecule of unknown structure, whereas the mass peak of 839 Da was attributed to a dimer complexed with a Na^+ ion. Further

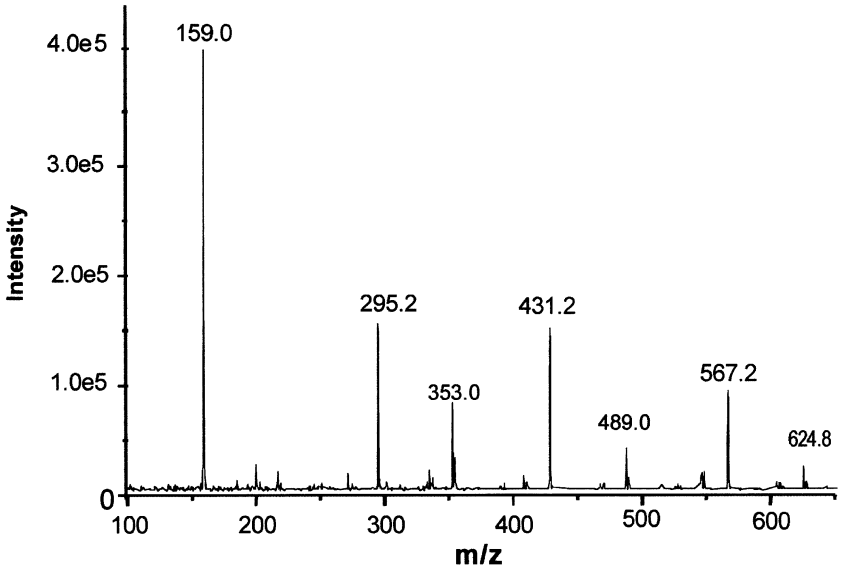

Fig. 3. ESI mass spectrum of the MCS-L04 factor in the positive ion mode.

mass peaks observed at 545 and 567 Da were not assigned [24], but can now be attributed to the MCS octamer with a Na^+ ion. For EDLF from human placenta, a 409 Da molar mass ion was reported [25], and the same mass ion is present in the MS spectrum of the EDLF from human plasma [26]. The molar mass ion of 625 Da for the EDLF factor isolated from pig urine which could not be assigned [27] may well correspond to the 624.8 Da peak of Fig. 4.

The ESI-MS spectrum of the MCS factors recorded in negative mode is shown in Fig. 4. The mass ion peaks (m/z) correspond to the negatively charged complexes of the MCS hexamer or of the octamer complexed with the trifluoracetate anion in various ratios. Thus, the 249.0 Da peak corresponds to $[(C_3O_2)_6+3CF_3COO^-]/3$, the 384.8 Da peak to $[2(C_3O_2)_6+3CF_3COO^-]$ the 521.0 Da peak to the $[(C_3O_2)_6+CF_3COO^-]$ and the 656.8 Da peak to the $[(C_3O_2)_8+CF_3COO^-]$ mass ion.

From a first quantitative analysis of the peak intensities a ratio of 65:35 was determined between the cyclohexamer/octamer forms of the MCS factors in the ESI-MS spectra. The intensity of the 295.2 and 159.0 Da mass ion peaks are less significant because they are assumed as multiple charged ions which may result both from cyclohexamer and -octamer. Due to the complex processes in the ionization chamber the preliminary composition data will be investigated in further studies.

3.3. Inhibitory potencies of the MCS factors

The lipophilic precursor is assumed to be a randomly structured polycarbonylic product that probably is produced by polyketide-synthase enzymes of ubiquitous distribution. Malonyl-coenzyme-A is known as building block of polyketides and carbon suboxide is actually the bis-anhydride of malonic acid. The purified lipophilic precursor inhibits the Na,K-ATPase with an IC_{50} of 0.5–2.5 μg/ml, while upon activation a significantly enhanced potency ($IC_{50}=0.015$ μg/ml) was observed. By the alkaline treatment of the polyketide precursor it is presumably transformed into the sodium salt of some open chain keto-enolic forms (Fig. 5). These are in turn transformed by treatment with 1 M HCl to reach a pH value between 8 and 9.6 into reactive intermediates which are entering subsequent condensation processes in the weak basic pH range with generation of the highly active MCS factors. The very low UV absorbance of the MCS factors in the 200–215 nm range excludes the formation of aromatic condensation products even as reaction intermediates. By a too rapid admixture of the acid sometimes an amorphous inactive gel of inorganic nature (inert to concentrated sulfuric acid) is formed. The scheme in Fig. 5 summarizes the steps assumed for the activation procedure.

Small but significant differences in the inhibition potencies of the resulting highly active MCS factors were observed (Fig. 6) when the probe was kept upon activation with excess sodium ions (e.g. MCS-402) or when these were removed with an ion exchanger resin. While a "sodium-free" preparation (e.g. MCS-L04) is capable of inhibiting the enzyme quantitatively, the MCS-402 preparation inactivates only 86% of the enzyme even at concentrations above 1 μM. Whether the remaining 14% enzymatic activity in the case of MCS-402 was produced by a slow turnover of (all) pumps or by an "inert" subpopulation of the Na,K-ATPase that could not be assessed so far.

Inhibition of rabbit Na,K-ATPase by the two MCS probes (MCS-L04, MCS-402) is compared in Fig. 6 to the inhibitory potency of ouabain. The concentration-dependent inhibition of the enzyme activity, E_A, was fitted by the Hill function $E_A(c)=E_A(0)/(1+(K_I/c)^{-nH})$, where $E_A(0)=1$ (normalized to the specific activity of the uninhibited enzyme), K_I is the inhibitory constant, corresponding to the half-inhibitory concentration of the compound added, and n_H is

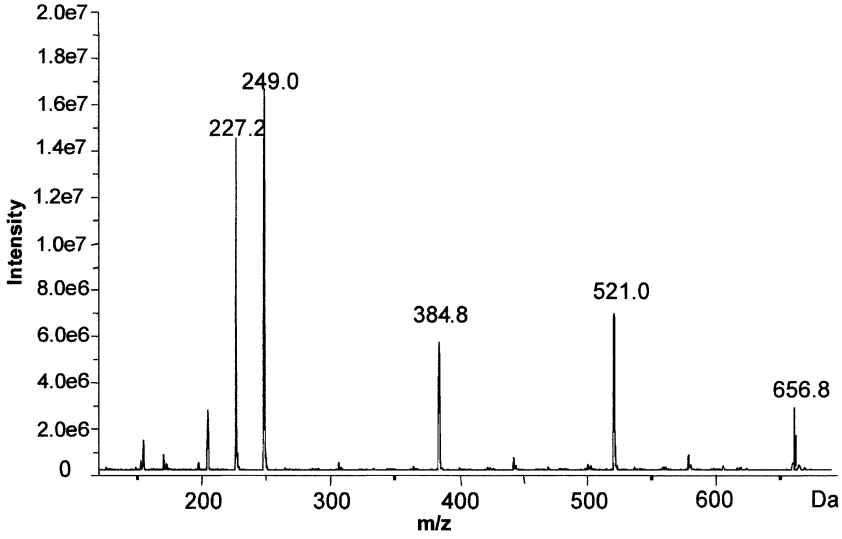

Fig. 4. ESI mass spectrum of the MCS-L04 factor in the negative ion mode.

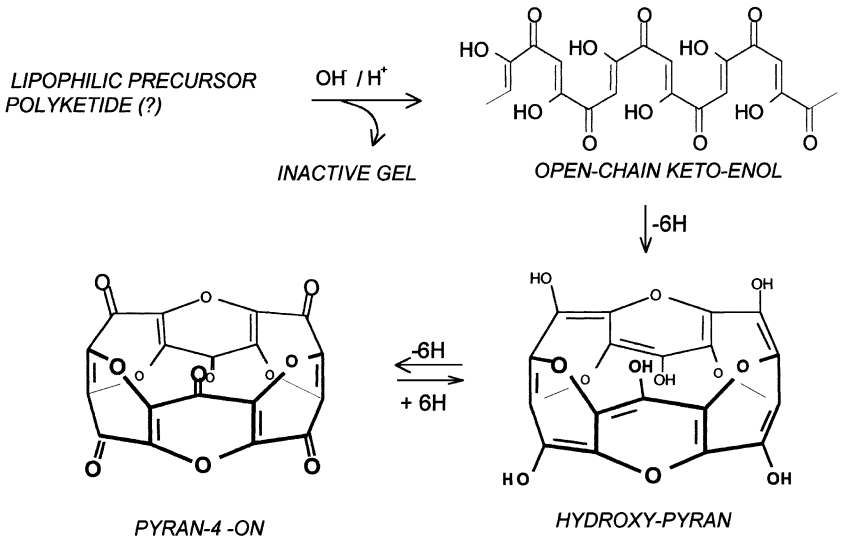

Fig. 5. Scheme for the preparation of the active MCS factors through the base/acid treatment of the lipophilic precursor form and the presumed intermediates.

the Hill coefficient. The inhibitory potencies of MCS-L04 (K_I = 38 nM) and MCS-402 (K_I = 24 nM) are about 26 or 40 times higher than that of ouabain (K_I = 1 μM). While the concentration-dependency of the MCS preparations could be fitted by a single binding isotherm, i.e. $n_H = 1 \pm 0.05$, in the case of ouabain, a higher value of n_H, i.e. 1.3, fitted best. This aspect will be the subject of further investigations focused on the inhibition mechanism (paper in preparation).

3.4. Inhibition of Na,K-ATPases from different tissues with MCS factors

It is well known that different isozymes of the Na,K-ATPase exhibit different affinities for ouabain [3]. A standard preparation of lower binding affinity for cardiac glycosides can be isolated from rat kidney [19]. The inhibition potencies of various MCS preparations were determined in parallel with rabbit and rat enzyme. As shown in Fig. 7, with MCS factors, no significant discrimination between the two enzymes could be obtained. The normalized specific enzyme activity was plotted against the concentration of the MCS factor (MCS-A02) and fitted with a single binding isotherm. The K_I values of 22.5 nM (rabbit) and 22.3 nM (rat) correspond to a ratio of K_I (rat)/K_I (rabbit) = 1. When fitted with a Hill function, the Hill coefficient with this MCS preparation was in the order of 1.3 (rat) to 1.8 (rabbit). In the case of another MCS preparation (MCS-A02), the ratio of K_I (rat)/K_I (rabbit) was 1.6 (data not shown). This would indicate that MCS preparations do not exhibit significant isozyme specificity, while the K_I (rat)/K_I (rabbit) determined in parallel for the same Na,K-ATPase enzyme preparations with ouabain was ~ 20 (data not shown). On the other hand, the various MCS preparations were found to differ in the Hill coefficient to some extent.

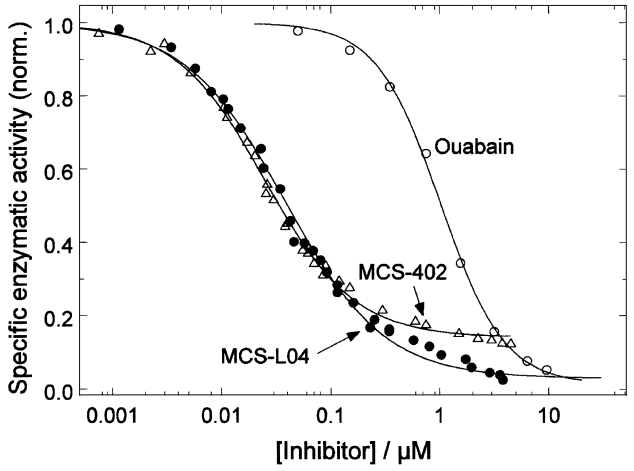

Fig. 6. Concentration dependent inhibition of Na,K-ATPase from rabbit kidney by differently activated MCS factors: Na^+ containing (MCS-402) and Na^+ free (MCS-L04) in comparison with ouabain. The concentration axis refers to the total inhibitor concentration.

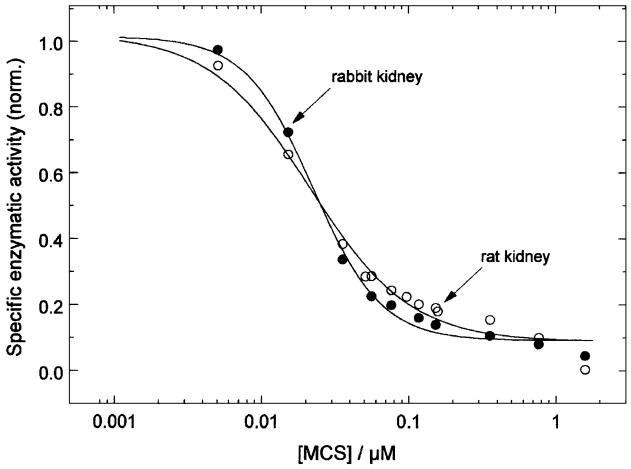

Fig. 7. Comparison of the inhibitory activity of the MCS-A02 factor on Na,K-ATPase isolated from rabbit and rat kidney.

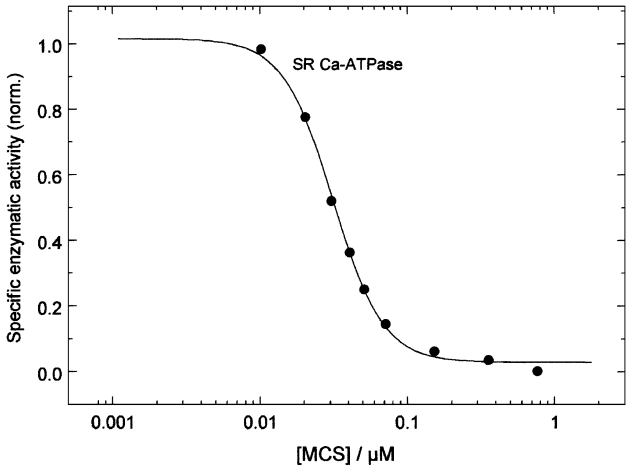

Fig. 8. Concentration-dependent inhibition of SR Ca-ATPase from rabbit by the MCS-A02 factor.

3.5. Inhibition of SR Ca-ATPase with MCS factors

Inhibition of SR Ca-ATPase with the MCS-A02 factor is shown in Fig. 8. The K_I of 31.3 nM was in the same order as that obtained with Na,K-ATPase. However, when fitted with a Hill function, the Hill coefficient, $n_H = 2.56$, was significantly higher than in the case of the Na,K-ATPases. This indicates that a different inhibitory mechanism may occur.

Bindings of inhibitors may induce conformational changes of the whole enzyme or of protein domains as recently assumed for some lipophilic, hydroxy-containing inhibitors of the SR Ca-ATPase [28]. Common structural elements of these molecules (ellagic acid, curcumin, diethylstilbestrol, etc.) are two or more phenolic hydroxy groups located on opposed parts of their structural frame.

Plant-derived poly-phenolic and flavonoid compounds and their synthetic analogs were identified as inhibitors of plasma membrane Ca-ATPase [29]. A similar polyphenolic structure is proper to hypericin, the protein kinase inhibitory substance from St.-John's-wort, recently identified as potent inhibitor of Ca-ATPase in lymphoid cells, too [30].

The Ca-ATPase inhibitory potency of the investigated polyphenolic compounds is with the exception of two synthetic eosin derivatives in the range of 10–1000 μM range [29], which is significantly lower than the inhibitory potency of the MCS factors.

4. Conclusions

Structurally, the new class of natural compounds isolated from *Helleborus purp.* are cyclo-oligomers of the inorganic carbon suboxide with a high tendency to self-association as well observed in the HPLC behavior. The MCS cyclohexamer, $(C_3O_2)_6$, with a molar mass of 408.2 Da was found to be the predominant component with about 2/3 of the contents, whereas the cyclooctamer $(C_3O_2)_8$ is present in lower amounts. Moreover, as evidenced by the ESI-MS spectra, the MCS factors show a tendency to form complexes with sodium and other ions.

The experimental data show that the MCS factors are potent and species-independent inhibitors of the Na,K-ATPase and are also capable of inhibiting the SR Ca-ATPase. Since the kinetics were always of the type of a binding isotherm with a well-defined half-saturating concentration, interactions with some specific sites of the protein have to be assumed. In contrast to cardiac glycosides, the MCSs do not discriminate significantly between rabbit and rat enzyme. This observation may possibly be explained by different mechanisms of interaction. The MCS inhibitors may interact with multiple structural elements of the protein including the ouabain binding site, but in a way which is not affected by the different amino acid compositions of rabbit and rat enzyme. To gain further insight into the mode of binding, functional analyses are in progress.

The still open question of the chemical nature of the endogenous inhibitor(s) of the Na,K-ATPase may possibly be tackled by the biochemical properties of the MCS inhibitors which share a high degree of similarity with EDLFs in their analytical properties. Variations in the inhibitory activity, as observed here for the MCS derivatives, have often been noticed for EDLF probes [12] and this observation suggests the existence of a structural equilibrium between variously active forms. Since MCS factors are also found in other plants and in bacteria (unpublished results), the investigation of their physiological activation and of their inhibitory mechanism may possibly answer the crucial question whether this class of compounds participates in the regulation of the Na,K-ATPase and possibly of other types of ATPases, too.

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