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Identification of the reaction products of (2′-5′)oligoadenylate synthetase in the marine sponge

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Previously we reported on the presence of a high (2′-5′)oligoadenylate synthetase activity in the marine sponge *Geodia cydonium* [Kuusksalu, A., Pihlak, A., Müller, W. E. G. & Kelve, M. (1995) *Eur. J. Biochem. 232*, 351−357]. The presence of (2′-5′)oligoadenylates [(2′-5′)A] in crude sponge extract was shown by radioimmunoassay and by their HPLC comigration with authentic (2′-5′)A oligomers. In addition, the sponge (2′-5′)oligoadenylates displayed biological activity, as determined by inhibition studies of protein biosynthesis in rabbit reticulocyte lysate. In the present study individual (2′-5′)oligoadenylates synthesized by sponge enzyme were separated by HPLC. The exact composition of every oligonucleotide peak eluted was determined by matrix-assisted laser-desorption-ionization mass spectrometry (MALDI-MS) analysis. The 2′-5′ phosphodiester bond in oligoadenylates was verified by NMR analysis. Based on the high concentration of (2′-5′)A oligomers in *G. cydonium* and their similarity with those found in mammals we propose that the (2′-5′)A system is involved in a cytokine-mediated pathway and/or in a protection system against viruses, present in the marine environment.

Keywords: (2′-5′)oligoadenylate; *Geodia cydonium*; matrix-assisted laser-desorption-ionization MS; NMR.

Sponges (Porifera) are the simplest multicellular animals, which have existed since the Proteozoic period [1]. In the past few years several cDNA/gene sequences have been isolated and characterized from sponges, especially from the marine demosponge *Geodia cydonium*. Analyses revealed that sponges contain proteins of the extracellular matrix/basal lamina (e.g. integrin receptor [2], collagen [3] or galectin [4, 5]), cell-surface receptors (tyrosine kinase receptor [6]), elements of the sensory system (crystallin [7], metabotropic glutamate receptor [8]) and homologs/modules of an immune system (immunoglobulin-like domains [9], scavenger receptor cysteine-rich domains and short consensus repeats [10], Rh-like protein [11]). These molecules were found to display high similarity to sequences from members of higher metazoan phyla [12].

Recently a cDNA encoding a putative cytokine, the endothelial monocyte-activating polypeptide [13], was identified in *G. cydonium* [14]. The physiological role of this factor in sponges is not known. One pathway in mammalian organisms which is controlled by cytokine(s) is the (2′-5′)oligoadenylate [2′-5′(A)] system. It regulates the RNA degradation pathway and is induced by interferons [15]. In mammals, the (2′-5′)A system is an important component of the cellular defence mechanism against virus infection induced by interferons [16]. The key enzyme of the pathway, (2′-5′)A synthetase [17−19], converts cellular ATP to a family of unusual, short 2′-5′-linked oligoadenylates (2′-5′)A carrying a triphosphate moiety at their 5′ end (general formula: pppA(2′p5′A)$_n$, usually $1 \leqslant n \leqslant 6$). (2′-5′)A synthetase exists in several isoforms that occupy different intracellular compartments [20−22]. Its enzymatic product, (2′-5′)A, functions as an allosteric activator of a latent endoribonuclease (RNase L) [23, 24], which degrades single-stranded viral and cellular RNA. (2′-5′)A oligomers are rapidly metabolized by 2′-phosphodiesterase [25, 26] and/or 2′,3′-exoribonuclease [27, 28].

Very little is known about the occurrence of the (2′-5′)A system in animals which are phylogenetically older than mammals. The activity of a (2′-5′)A synthetase has been detected in birds and reptiles [29]; a putative (2′-5′)A synthetase has been partially purified from tobacco [30]. Moreover, (2′-5′)A binding protein(s) have been found in low amounts in amphibia [29] and in potato leaves [31]. (2′-5′)A molecules have even been reported in *Escherichia coli* [32] and in some other bacteria and yeasts [33]. These findings describe one step of the (2′-5′)A system; no reports about a complete (2′-5′)A system in organisms other than mammals have been published yet. Likewise, in invertebrates or even in protozoa, the existence of a functional (2′-5′)A system is unknown.

Recently, we have discovered high (2′-5′)A synthetase activity in the marine sponge *G. cydonium* [34]. (2′-5′)oligoadenylates isolated from crude sponge extract competed with authentic chemically synthesized (2′-5′)A for binding to polyclonal antiserum against (2′-5′)A [35]. Individual (2′-5′)oligonucleotides synthesized with partially purified sponge (2′-5′)A synthetase were subjected to HPLC and identified by their comigration with mo-

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Abbreviations. (2′-5′)A, (2′-5′)oligoadenylates; (p$_x$A$_n$), (2′-5′)oligoadenylates containing *x* phosphate groups and *n* adenylate residues; (2-5)A synthetase, (2′-5′)oligoadenylate synthetase; poly(I) · poly(C), polyriboinosinic acid · polyribocytidylic acid; MALDI-MS, matrix-assisted laser-desorption-ionization mass spectrometry.

Enzymes. (2′-5′)oligoadenylate synthetases (EC 2.7.7.–); ribonuclease L (EC 3.1.27.–); 2′-phosphodiesterase (EC 3.1.4.–); 2′,3′-exoribonuclease (EC 3.1.13.–); calf intestinal phosphatase (EC 3.1.3.1).

lecular markers for (2′-5′)A oligomers. In this study we present data on the direct identification by matrix-assisted laser-desorption-ionization mass spectrometry (MALDI-MS) analysis of the total (2′-5′)oligoadenylate pool synthesized by the sponge (2′-5′)A synthetase. Furthermore, NMR technique was used for verification of the 2′-5′ phosphodiester bond in oligoadenylates synthesized by the sponge enzyme.

MATERIALS AND METHODS

Sponge. Live specimens of the marine sponge *G. cydonium* (Porifera, Demospongiae, Geodiae) were collected in the Northern Adriatic Sea near Rovinj (Croatia), cut to pieces and immediately frozen in liquid nitrogen.

Cell extracts. Frozen sponge tissue was mechanically broken in liquid nitrogen using mortar and pestle and homogenized with an equal amount (mass/vol.) of the polymerase assay buffer (buffer A) consisting of 20 mM Tris/HCl, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40 and 5% glycerol. The mixture was centrifuged (10 min; 10 000×*g*, 4 °C), the supernatant was collected and frozen immediately. The protein concentration was 12 mg/ml.

Preparative synthesis of (2′-5′)A oligomers. Synthesis of (2′-5′)A oligomers using sponge (2′-5′)A synthetase was carried out with the partially purified enzyme bound to polyriboinosinic acid · polyribocytidylic acid [poly(I) · poly(C)] membrane as described [36]. Poly(I) · poly(C) membrane for binding and activation of the (2′-5′)A synthetase was prepared by hybridizing poly(I) to poly(C), previously immobilized to Hybond C membrane (Amersham). Standard northern blotting hybridization method in the presence of formamide was applied [37]. The concentration of poly(I) and poly(C) in the hybridization reaction was 10 mg/ml, the washing steps were performed in the presence of 0.001% diethylpyrocarbonate.

For the synthesis of (2′-5′)A oligomers, three 1 cm^2 pieces of poly(I) · poly(C) membrane were washed three times with buffer A and dried for 10 min on filter paper. The enzyme was immobilized from 3 ml of sponge extract at room temperature with gentle shaking for 30 min. The membrane with bound (2′-5′)A synthetase was washed three more times with buffer A and then dried for 10 min. The reaction mixture contained 1 mM ATP, 30 mM Tris/HCl, pH 7.6, 100 mM KCl and 5 mM $MgCl_2$. The synthesis was performed in 24-well plate wells (Nunc) at room temperature for 10 h. During this period almost all of the ATP was converted into (2′-5′)A oligomers. The synthesis mixture together with the reaction products were pipetted into a microcentrifuge tube. The membrane used for synthesis was washed with water and the eluate was combined with the mixture; this material was concentrated in a vacuum centrifuge.

High performance liquid chromatography. *Sample preparation for MALDI-MS analysis.* The (2′-5′)A oligomers synthesized were applied to the HPLC apparatus (Du Pont) equipped with a C_{18} reverse-phase column (Supelcosil LC 18, 30 cm) and separated as described [38] applying a 0 to 30% methanol gradient in 50 mM ammonium phosphate, pH 7.0, for 30 min. Fractions were collected manually by measuring absorbance at 254 nm and concentrated in vacuum centrifuge.

The fractions were rechromatographed for desalting in 100 mM triethylammonium acetate, pH 7.0, using a 10 to 30% acetonitrile gradient (30 min). Fractions were collected and re-evaporated three times in vacuum centrifuge (dissolving them between the concentration steps in double-distilled water) to eliminate the traces of triethylammonium acetate, and then dissolved in water. The concentrations of the (2′-5′)A oligomers were calculated by their absorbance at 254 nm.

Sample preparation for NMR analysis. The triphosphorylated (2′-5′)A dimer, separated and desalted as described above, was treated with calf intestinal phosphatase (Boehringer Mannheim) for 5 h at 37 °C [34]. The resulting dephosphorylated core dimer was re-chromatographed in a methanol gradient as described above. The product was lyophilized and redissolved in deuterated water.

MALDI-MS analysis. Mass spectrometric analysis of (2′-5′)oligoadenylates synthesized by the sponge enzyme, was conducted with a home-built matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer in linear configuration and with delayed pulsed extraction. 3-Hydroxypicolinic acid (Aldrich Chemicals) was used as the matrix with ammonium tartrate (synthesized from tartaric acid and ammonium carbonate) to suppress alkaline metal adducts. The matrix solution was prepared by dissolving 50 mg of 3-hydroxypicolinic acid and 9.2 mg of ammonium tartrate in 1 ml H_2O/acetonitrile (1:1 by vol., HPLC grade). 1-µl aliquots of concentrated fractions obtained after chromatographic separation were mixed with 5 µl of matrix solution. Aliquots of 1 µl of the resultant mixture were deposited on a stainless steel target and air-dried before introducing into the mass spectrometer.

NMR analysis. An approximately 0.5 mM solution of the dephosphorylated (2′-5′)A dimer was measured at 500 MHz at 20 C on a Bruker AMX spectrometer while dissolving the sample in ^2H_2O without any previous treatment of nucleotides for their enrichment with ^2H_2O. Several hundreds of scans were performed to observe NMR signals from this dinucleotide solution.

RESULTS

MALDI-MS analysis. MALDI-MS analysis was applied to samples obtained after the HPLC separation on a C_{18} column; the separation was performed in phosphate buffer using a methanol gradient. The standard separation curve is shown in Fig. 1. Overlapping peaks were collected manually and subjected to rechromatography in triethylammonium acetate buffer. Oligoadenylates eluted as broad tailing peaks in the triethylammonium acetate containing an acetonitrile gradient. However, this step was performed to change the buffer into a volatile one rather than for further separation.

As an example, the MALDI-MS analysis of the sample no. 8, corresponding to 16.4 min. retention time (shown in Fig. 1), is given in Fig. 2. Rechromatography of this fraction in triethylammonium acetate buffer with acetonitrile as eluent revealed three partially resolved peaks with respective retention times of 17.8, 20.2 and 21.6 min, which were collected and analyzed separately. In MALDI spectra three main compounds could be identified which were eluted in triethylammonium acetate buffer in retention order pA_4, p_3A_5 and p_3A_6. However, all tetra-, penta- and hexa-adenylates could be found with some intensity (Fig. 2b and c). The protonated oligoadenylate peaks were accompanied by smaller alkali metal adduct peaks on the high mass side. In all spectra, smaller p_2A_n peaks preceded p_3A_n peaks, and in the same way smaller A_n peaks preceded pA_n peaks. This could be a result of dephosphorylation of the polyadenylates in the MALDI sample preparation or in the MALDI process itself. No significant breakdown of the polyadenosine moiety was detected in the MALDI process.

In MALDI sample preparation different components of a mixture can crystallize in different areas of the sample, depending on when the drying solution becomes saturated for them. Because of this, spectra from different spots on the sample were compared and less than 30% difference in the intensity ratios of

Table 1. Identification of reaction products synthesized by the (2′-5′)A synthetase from *G. cydonium* from exogenous ATP by MALDI-MS analysis. Main components are marked in bold. Chromatography 1, HPLC analysis with C_{18} column using 50 mM phosphate, pH 7.0 and a 0 to 30% methanol gradient (running time; 30 min). Chromatography 2, HPLC C_{18} column analysis with the 100 mM triethylammonium acetate, pH 7.0, and a 10 to 30% acetonitrile gradient (running time, 30 min).

Sample no.	Retention time		M_r (experim.)	Oligoadenylate (p_xA_n)	M_r(calc.)
	Chromatography 1	Chromatography 2			
	min				
1	8.7	7.7	836.8	p_3A_2	837.4
2	9.4	6.3	756.8	p_2A_2	757.4
		7.5	757.6	p_2A_2	757.4
			836.8	p_3A_2	837.4
3	12.7	6.7	677.6	p_1A_2	677.4
4	13.2	12.3	1086.0	p_2A_2	1086.6
			1165.8	p_3A_3	1166.6
5	14.9	17.0	1415.3	p_2A_4	1415.8
			1495.4	p_3A_4	1495.8
6	15.3	14.0	1006.7	p_1A_3	1006.6
		18.0	1006:7	p_1A_3	1006.6
			1415.4	p_2A_4	1415.8
			1495.6	p_3A_4	1495.8
7	15.7	14.2	1006.4	p_1A_3	1006.6
		18.0	1006.7	p_1A_3	1006.6
			1415.8	p_2A_4	1415.8
			1495.8	p_3A_4	1495.8
			1824.8	p_3A_5	1825
		19.2	1745.0	p_2A_5	1745.0
			1825.2	p_3A_5	1825
8	16.4	17.8	1335.9	p_1A_4	1335.8
			1496.1	p_3A_4	1495.8
			1665.1	p_1A_5	1665
			1745.0	p_2A_5	1745
			1824.8	p_3A_5	1825
		20.2	1335.8	p_1A_4	1335.8
			1495.7	p_3A_4	1495.8
			1664.9	p_1A_5	1665
			1745.1	p_2A_5	1745
			1825.0	p_3A_5	1825
			2153.8	p_3A_6	2154.2
		21.6	1335.4	p_1A_4	1335.8
			1824.6	p_3A_5	1825
			1994.1	p_1A_6	1994.2
			2074.2	p_2A_6	2074.2
			2153.5	p_3A_6	2154.2
9	16.8	16.5	1335.9	p_1A_4	1335.8
			1665.6	p_1A_5	1335.8
		18.6	1335.7	p_1A_4	135.8
			1495.6	p_3A_4	1495.8
			1664.8	p_1A_5	1665
			1825.0	p_3A_5	1825
		20.4	1335.7	p_1A_4	1335.8
			1495.9	p_3A_4	1495.8
			1665.2	p_1A_5	1665
			1745.1	p_2A_5	1745
			1825.0	p_3A_5	1825
			1994.5	p_1A_6	1994.2
			2074.2	p_2A_6	2074.2
			2154.4	p_3A_6	2154.2
			2483.6	p_3A_7	2483.4
		>22.0	1335.2	p_1A_4	1335.8
			1664.4	p_1A_5	1665
			1744.5	p_2A_5	1745
			1824.6	p_3A_5	1825
			1993.7	p_1A_6	1994.2
			2073.6	p_2A_6	2074.2
			2482.8	p_3A_7	2483.4
			2812.2	p_3A_8	2812.6

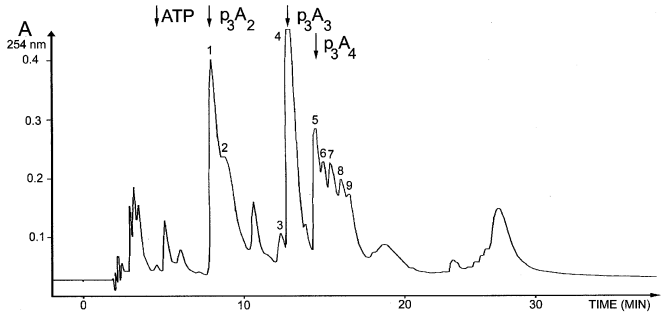

Fig. 1. Reverse phase HPLC elution profile with methanol gradient resolving the reaction products synthesized by the (2′-5′)A synthetase from *G. cydonium* using exogenous ATP.

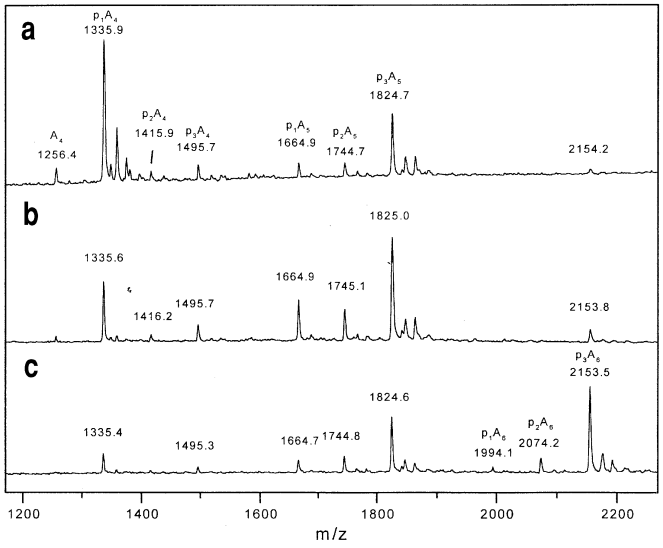

Fig. 2. MALDI-MS spectra of sample no. 8 (see Table 1) with the retention times in triethylammonium acetate 17.8 min (a), 20.2 min (b) and 21.6 min (c), respectively.

different lines within a spectrum was found. So we believe that the spectra give at least a qualitatively correct picture of the composition of collected chromatographic peaks.

Table 1 summarizes the full identification by MALDI-MS method of the compounds in all HPLC fractions collected.

NMR analysis. ^{1}H NMR studies of nucleotides are typically performed at concentrations of tens of millimoles per litre [39–43], in some cases up to more than 100 mM [42]; others have used a 600-MHz instrument in which about 5 mM solution has been measured [43]. As a rule, nucleotides are pretreated with cation-exchange resins, lyophilized several times from highly enriched ^2H_2O and the final solution is prepared in 99.9% ^2H_2O. In the present study, a 0.5 mM solution was measured without any previous treatment simply by solving the sample in ^2H_2O. Several hundreds of scans were needed to observe NMR signals from this dinucleotide solution. The residual signal from HDO was more than 1000-times stronger than the signals from the nucleotide. Therefore, presaturation of HDO signal was used. Good suppression of the HDO signal was obtained, and even the multiplet from $H_{2'}$ of Np- residue, which appeared about 100 Hz from the HDO resonance, was observed.

The resulting spectrum unambiguously confirmed the structure of A2′-5′A, as compared with the published data on this nucleotide [39]. Comparison of data from two measurements (Table 2) showed marked differences in some chemical shifts (up to $\pm$60 ppb), but spin-spin coupling values, among them the ^{31}P nucleus, were practically the same. This is the proves the existence of the 2′-5′ phosphodiester bond in organisms other than mammals, using physical methods. A structure of A3′-5′A was ruled out by the reported chemical shift difference of H1′ protons equal to 0.11 ppm [44] (in the present study 0.326 ppm) and their coupling constants (3.2 Hz and 3.5 Hz [44]). The presence of A5′-5′A was ruled out by symmetry considerations.

Table 2. Comparison of ^{1}H chemical shifts and spin-spin coupling constants of A2′-5′A in 57 mM [40], 0.5 mM and infinitely diluted [45] heavy water solution.

Fragment	Proton	0.5 mM solution		57 mM solution[c]		Infinite dilution[d]	
		δ[a]	J[b]	δ	J	δ	J
Np-	1′	6.560	4.5	6.560	4.7	6.56	4.5
	2′	5.574	5.0 9.3(P)	5.569	5.0 9.3(P)		
	3′	5.061	4.8	5.084	4.6		
	4′	4.650	2.4 3.1	4.690	2.4 3.3		
	5′	4.222	13.1	4.279	13.0		
	5″	4.119		4.176			
	2	8.891	s	8.136	s	8.15	
	8	8.615	s	8.587	s	8.59	
-pN	1′	6.236	3.7	6.230	3.7	6.22	2.8
	2′	4.715	4.9	4.734	4.9		
	3′	4.746	5.6	4.744	5.9		
	4′	4.556	1.8 2.4 2.3(P)	4.561	1.8 2.4 2.5(P)		
	5′	4.469	11.6 3.3(P)	4.480	11.5 3.5(P)		
	5″	4.354	3.7(P)	4.333	3.6(P)		
	2	8.600	s	8.540	s	8.58	
	8	8.431	s	8.405	s	8.41	

[a] Extrapolation to infinite dilution chemical shift of H-1′ of Np- [45] has been used for the reference.
[b] Coupling constants for protons with lower number are not repeated, spin-spin coupling to P is marked by (P).
[c] From [39], reported data interpolated to 20 °C form 9 °C and 39 °C values. For comparison purposes chemical shifts of H-1′ of Np- are taken as equal. It has been shown that H-1′ of Np- has the smallest concentration effect [44].
[d] From [44], interpolated to 20 °C from 4 °C and 30 °C values.

DISCUSSION

Sponges (phyla Porifera) are the simplest multicellular animals and have existed since the Proteozoic period [1]. As summarized above, these animals have key structural and functional elements, found also in higher metazoan phyla. In the first approach to elucidate a cytokine-related pathway similar to that in mammalian organisms, the (2′-5′)A system was studied in the sponge *G. cydonium*. Previously, we reported that this animal contains one enzyme of this system, the (2′-5′)A synthetase [34]. Suprisingly, the enzyme which is usually induced by interferons in mammalian cells is very active in sponge cells [34]. In the present study it was shown by physical methods that the (2′-5′)oligoadenylates synthesized by the sponge enzyme are identical to those in mammalian cells, that is, they have 2′-5′ phosphodiester bonds between the adenosine residues and carry triphosphate groups at their 5′ ends. The latter can be seen in Table 1 from molecular mass data estimated by MALDI-MS method.

MALDI-MS has been shown to be ideally suitable for identification of small oligonucleotides [45]. Two important advantages of MALDI-MS, the sensitivity and ability to analyze complex mixtures, allow the identification of any oligonucleotide chromatography pattern within seconds. These advantages are now being utilized to sequence biopolymers [46, 47]. MALDI-MS has also been applied to large-scale DNA sequence analysis [48−54]. The accuracy in mass provided by delayed extraction MALDI-MS is fully adequate for this kind of analysis. In experiments described here, the discrepancy between calculated and experimental values of molecular masses never exceeded one molecular mass unit. This allows us to identify unambigously (2′-5′)oligoadenylates with different phosphorylation state.

Reverse-phase chromatography in a methanol gradient buffered with ammonium phosphate is a suitable method for separation of these reaction mixtures. Retention times for oligoadenylates carrying the 2′-5′ bond and the 3′-5′ bond are sufficiently different [38] and highly reproducible, which allows application of external standards for peak identification.

In our experiments, desalting was needed to remove disturbing phosphate ions from the samples before MALDI-MS measurements [45]. Table 1 shows that fractions collected in standard chromatography were split into several peaks by re-chromatography in a triethylammonium acetate buffer. This means that substances which have very similar retention times in pure reverse-phase chromatography, have an altered elution order in the triethylammonium acetate/acetonitrile system due to the ion-pairing properties of the triethylammonium ions [55]. The retention times for oligomers in triethylammonium acetate chromatography were not quite constant within a series of injections, in contrast to the phosphate/methanol system. Nevertheless, chromatography in the triethylammonium acetate buffer proved to be an efficient and rapid method for the preparation of salt-free oligoadenylates.

We found diphosphorylated and monophosphorylated (2′-5′)oligoadenylates in MALDI-MS analysis in addition to the triphosphorylated (2′-5′)oligoadenylates. Dephosphorylation of triphosphorylated (2′-5′)oligoadenylates starts during solid-phase synthesis since peaks corresponding to p_2A_2 and pA_2 are present in primary HPLC separation (peaks 2 and 3 in Fig. 1, respectively), and may continue during sample handling. This is caused by the intrinsic lability of the triphosphate moiety. Dephosphorylation of oligonucleotides also occurs in MALDI-MS analysis as evidenced by the presence of a weak signal for A_4 in parallel to that for pA_4 (Fig. 2a, b). This obviously takes place in the MALDI-MS process after HPLC purification, because 5′-phosphates are usually stable in solutions, and A_4 and pA_4 could not comigrate in reverse-phase HPLC. In spite of difficulties rising due to degradation of the primary synthesis products, we were able to show clearly that sponge (2′-5′)A synthetase activity was capable of producing at least up to (2′-5′)octaadenylate triphosphates. Thus, the ability of sponge (2′-5′)A synthetase to synthesize long oligomers is analogous to mammalian (2′-5′)A synthetase activity.

The apparent peak in Fig. 1 between the peaks 2 and 3, which could not be assigned to any (2′-5′)oligoadenylate product by chromatographic mobility, was also analyzed by MALDI-MS. This peak was not a derivative from ATP, as revealed by MALDI-MS showing a spectrum which contained a series of signals separated by exactly 161 Da starting at 345 Da. This was probably an impurity eluted from the HPLC column or the nitrocellulose membrane.

The exact role of these unusual (2′-5′)oligoadenylates in sponge remains to be elucidated. However, the high concentration of these mediators [34] together with their similarity with those found in mammals, point to their important function in sponges. At present it can be postulated that the an (2′-5′)A system is involved in a cytokine-mediated pathway and/or in the protection system against viruses, present in the marine environment. The existence of cytokines or related molecules has been shown in sponges [14] and the presence of viruses in the marine milieu is well documented [56]. Studies are in progress to identify further components of the (2′-5′)A system in sponges and to elucidate the structure of the sponge (2′-5′)A synthetase gene(s).

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