MS-guided Fractionation as a Fast Way to the Identification of New Natural Products – MALDI-TOF-MS Screening of the Marine Sponge *Stylissa caribica*

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MALDI-TOF-MS was used for the first time as a direct screening method to analyse sponge tissue (*Stylissa caribica*). In order to validate this method the results were compared with a HPLC-ESI-MS analysis of the crude extract from the investigated sponge. Both methods showed a good agreement in their results, allowing for the rapid identification of 15 pyrrole-imidazole alkaloids.

Key words: Natural Products, Marine Sponge, Mass Spectrometry

Introduction

A common approach for the isolation of new bioactive natural products makes use of a bioassay-guided fractionation of an organic extract which has shown activity in a pre-screening [1]. The major disadvantage of this method is the lack of guarantee that the active component represents a new structure. A different approach to find new natural products employs the MS-guided fractionation of an entire organism. The MALDI-TOF-MS technique [2] allows to run this approach directly on a sample of the organism without extraction [3]. Here, we have applied this method for the first time to the tissue of a marine sponge (*Stylissa caribica*) [4]. This method would allow to accelerate the process of dereplication of secondary metabolites and furthermore to gain information on the distribution of seondary metabolites within an organism. In order to validate the analytical quality and the general application of this approach we have also investigated the sponge *Stylissa caribica* by the routinely used HPLC-ESI-TOF-MS method. This offered a direct comparison of the identified secondary metabolites by both methods.

Sponges of the families Axinellidae and Dictyonellidae belong to the best investigated marine invertebrates. Therefore, a lot of secondary metabolites have already been described in the literature. The most prominent natural products isolated from these sponges include the agelastatines [5], axinellamines [6], hymenialdisin [7], phakellins [8], hymenamides and phakellistatins [9]. It is therefore very important to know at a very early stage of the structure elucidation process whether a sponge contains novel natural products or not.

The MALDI-TOF-MS technique is usually applied to the structure elucidation of biomacromolecules [10]. Another application includes the investigation of bacteria with respect to their chemotaxonomy [11]. Von Döhren *et al.* proposed this method to directly analyse the secondary metabolism of intact organisms [3]. They applied MALDI-TOF-MS to toxic cyanobacteria, leaves of *Catharantus roseus*, and actinomycetes [12]. For the application of the MALDI-TOF-MS technique the sample has to be dissolved in a special matrix which is useful for ionization of the compounds. Two experimental approaches are possible: a) mixing the sample material (*e. g.* bacteria or sponge powder) with matrix solution directly on the MALDI target, or b) preparing a suspension of sponge powder in organic solvents and subsequent transfer of this solution on the MALDI target and mixing with matrix solution. The latter method was used here to analyse the sponge *Stylissa caribica*.

Results and Discussion

To apply the MALDI-TOF-MS method a quite simple sample preparation is required. The sponge tissue of *Stylissa caribica* was crushed with a mill and

Table 1. Comparison of selected detected compounds with MALDI-TOF-MS and HPLC-ESI-oTOF-MS from the sponge *Stylissa caribica*. MS spectra were only obtained in positive mode.[a,b]

	Detected compounds by		
m/z	MALDI-TOF-MS	HPLC-ESI-TOF-MS	
$[M+H]^+$		Retention (min)	Rel. peak area (%)[c]
308.0	debromostevensine[e] [13]	6.1	0.5
310.0	monobromoisophakellin[d] [14]	5.5	4.4
310.0	hymenidin (**1a**)[d] [15]	10.1	6.1
318.0	4-bromopyrrole-2-carboxy-*N*(ε)-lysine[d] [16]	8.4	3.6
324.0	*N*-methylbromo(iso)phakellin[e,f]	11.3	0.5
340.0	oxocyclostylidol (**2**)[d] [17]	3.1	1.3
346.0	4-bromopyrrole-2-carboxyarginine[d] [16]	7.8	3.2
385.9	stevensine (**3**)[d] [18]	8.8	45.6
387.9	oroidin (**1b**)[d] [19]	14.1	19.1
401.9	*N*-methyldibromoisophakellin[e] [20]	12.6	2.5
576.0	dibromopalau'amine[d] [21]	2.3	2.3
619.0	ageliferin (**4**)[d] [22]	14.9	0.4
765.4	hymenamide F (**5**)[e] [23]	14.8	0.4
824.8	tetrabromostyloguanidine (**6**)[d] [24]	13.7	2.7
827.4	hymenamide C[d] [25]	24.2	0.3

[a] Masses are given as monoisotopic masses; [b] due to the applied positive mode, 4,5-dibromopyrrole-2-carboxylic acid [20a] was not detected by MALDI-TOF-MS; [c] data obtained by UV detection (280 nm); [d] compound was identified by NMR and its high accuracy mass (ESI-TOF-MS); [e] compound was identified only on the basis of his high accuracy mass (ESI-TOF-MS); [f] for this compound the phakellin or isophakellin form cannot be distinguished by MS.

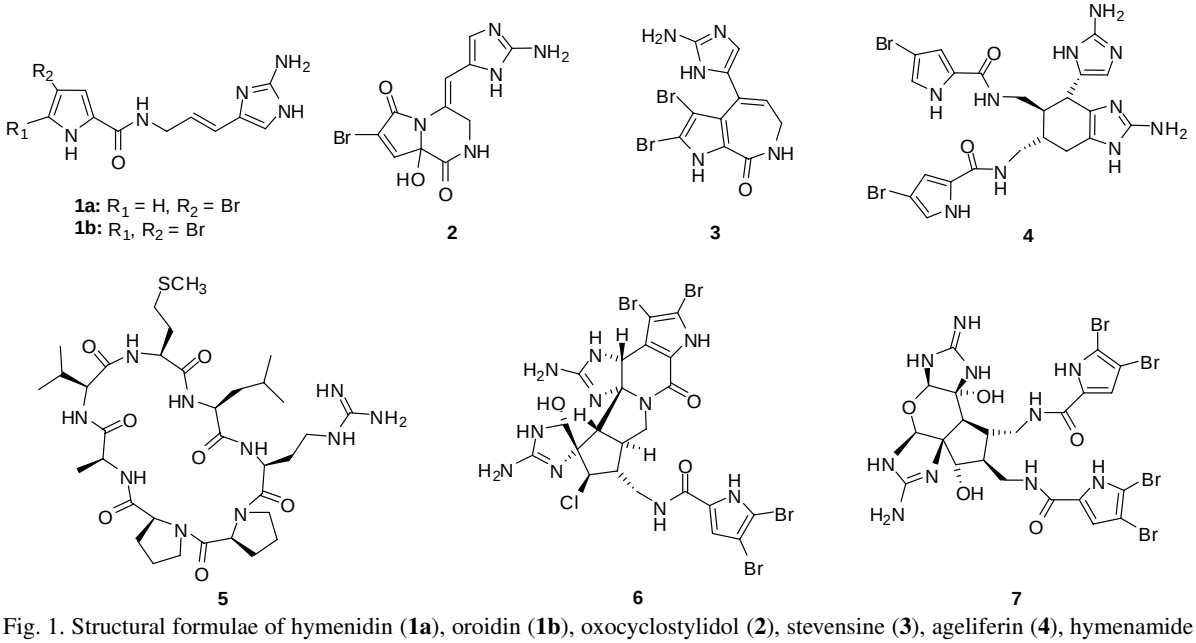

Fig. 1. Structural formulae of hymenidin (**1a**), oroidin (**1b**), oxocyclostylidol (**2**), stevensine (**3**), ageliferin (**4**), hymenamide F (**5**), tetrabromostyloguanidine (**6**), and massadine (**7**).

the resulting powder was suspended in a 1 : 1 mixture of MeCN and TFA (2.5 % in water). The sample was placed on a steel target using HCCA matrix. While making a crude extract includes overnight extraction, the sample preparation and MALDI measurements are possible in less than one hour. In order to exclude matrix effects, reference spectra of the MALDI matrix (HCCA) were acquired. The spectrum of the matrix did not show any overlapping with the area of expected masses. The MALDI-MS spectra of the sponge powder displayed several halogenated and non-halogenated compounds in the mass range from *m/z* = 300 to 900 (see Fig. 2 and Table 1).

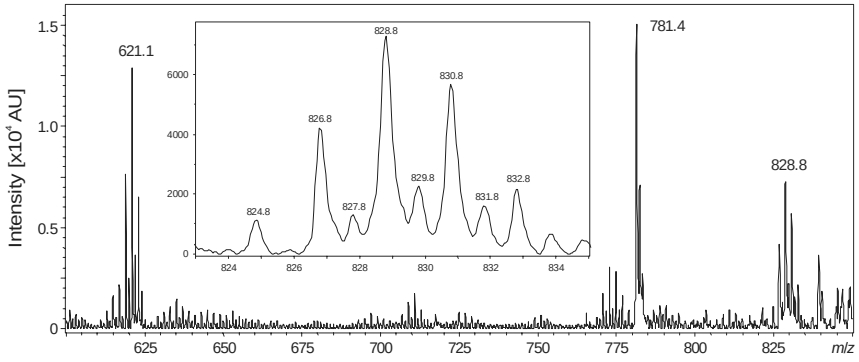

Fig. 2. Section of the MALDI-TOF-MS spectrum of sponge powder of *Stylissa caribica*. At *m/z* = 621.1 ageliferin (**4**) was detected, whereas at *m/z* = 781.4 a previously unobserved non-halogenated substance occurred. Tetrabromostyloguanidine (**6**) was detected at *m/z* = 828.8, the isotopic cluster (small section) is consistent with a Br_4Cl pattern.

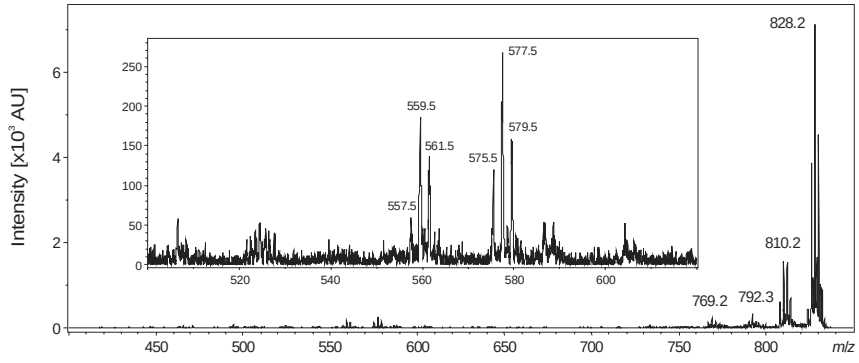

Fig. 3. Sections of the MALDI-TOF MS/MS spectrum (LIFT® technology) of tetrabromostyloguanidine (**6**). For the precursor ion at *m/z* = 828.2 a double loss of water (*m/z* = 810.2 and *m/z* = 792.3), a loss of guanidine (*m/z* = 769.2) and the specific loss of *m/z* = 250.7 (*m/z* = 577.5) was observed. The loss of *m/z* = 250.7 is specific for all pyrrole-imidazole alkaloids which include a non-cyclized 4,5-dibromopyrrole-2-carboxylic acid moiety. The isotopic pattern of the fragment peak at *m/z* = 810.2 (loss of water) is overlapped by a peptide fragment (*m/z* = 812.3) which is generated from its Na-adduct at *m/z* = 834.2. For selection of precursor ions for fragmentation using the LIFT® technology an interval of 8 amu (1 % of precursor ion mass) was chosen. This interval included the Na-adduct of the unknown peptide. For the LIFT® spectrum no nearest neighbour calibration was performed.

The acquired masses were compared with the results of routine HPLC-ESI-TOF-MS analyses of the crude extract of *Stylissa caribica*. The MALDI-TOF-MS and HPLC-MS measurements showed a good agreement with regard to the number of detected substances (see Table 1). In the low mass range (*m/z* = 300 to 700) all compounds detected by HPLC-MS were also recovered by MALDI-TOF-MS. For higher molecular masses the two methods showed a greater difference in the recovery of substances. Especially, the detection of oxidized dimeric pyrrole-imidazole alkaloids, *e. g.* massadine (**7**) [26] under MALDI conditions was not as effective as for smaller alkaloids. This conclusion did not apply to other substance groups with a similar molecular weight range (*m/z* exceeding 700). For instance, cyclic heptapetides of the hymenamide [9] group such as hymenamide C [25] and F (**5**) [23] could easily be detected under MALDI conditions. It is unlikely that the dimeric pyrrole-imidazole alkaloids are chemically unstable and decompose under MALDI conditions. Probably the occurrence of the hydrophilic amino-imidazole moieties in addition to hydroxyl groups causes high polarity of the metabolites. In comparison to massadine (**5**) the detected dimeric pyrrole-imidazole alkaloid tetrabromostyloguanidine (**6**) includes a chlorine atom instead of an additional hydroxyl group. The chlorine atom increases the lipophilic character of the molecule which may support the ionization using the HCCA matrix.

Fig. 3 shows the results of the MALDI-MS measurements of sponge powder. At *m/z* = 621.1, ageliferin (**4**) [22] was detected which was confirmed by NMR analysis after separation by chromatography. One of the oxidized dimeric pyrrole-imidazole alkaloids is tetrabromostyloguanidine (**6**) [24] which was recently isolated from this sponge. With this example first steps in structure elucidation were performed using MS/MS techniques. The isotopic pattern of the signal at *m/z* = 828.8 indicated a compound with one chlorine and four bromine atoms. Applying the MALDI-MS/MS technique (LIFT® technology) [27] a fragmentation typical for non-cyclized pyrrole-imidazole alkaloids appeared (see Fig. 3). The double loss of 18 amu proved at least one hydroxyl group, whereas the fragment at *m/z* = 577.5 (which corresponds to the loss of 251 amu) is a hint for a 4,5-dibromopyrrole-2-carboxylic acid moiety. This fragment is observed for all pyrrole-imidazole alkaloids containing a non-cyclized 4,5-dibromopyrrole-2-carboxylic acid moiety, *e. g.* oroidin (**1a**), sceptrin, ageliferin (**4**), and massadine (**7**). This example clearly indicates how fast structural information of secondary metabolites can be gained by MALDI-TOF-MS.

Conclusion

The direct MS analysis (MALDI-TOF-MS) of sponge tissue represents a fast approach for the dereplication of secondary metabolites without a time-consuming extraction of the organism. The comparison of the HR-ESI-MS and MALDI-TOF-MS analyses permitted to obtain information on the general application of the latter method. The results have shown that the information gained from MALDI-TOF-MS are in good accordance with data obtained by HR-ESI-TOF-MS techniques. Only in the region of higher molecular weights (here: 800 to 900 amu) the MALDI-TOF-MS technique was not able to detect all masses identified by HR-ESI-TOF-MS. Altogether, MALDI-TOF-MS in combination with spectroscopic methods allows the fast identification, isolation, and structure elucidation of new natural products from marine sponges and possibly other organisms. Furthermore, the MALDI technique would allow the analysis of specific compartments or organelles of an organism leading to information about the distribution of secondary metabolites within the organism.

Experimental Section

The samples of *Stylissa caribica* were frozen immediately after collection and kept at −20 °C until extraction. The freeze dried sponge samples of *Stylissa caribica* (94.7 g) were crushed with a mill and extracted at r. t. exhaustively with a 1 : 1 mixture of CH_2Cl_2/MeOH [16, 17].

HPLC-MS analyses were performed with an Agilent 1100 HPLC system and a Bruker Daltonics microTOF$_{LC}$ mass spectrometer. Separation was achieved by a Waters XTerra RP$_{18}$ column (3.0 × 150 mm, 3.5 μm) applying a MeCN/H_2O/HCOOH gradient (0 min: 10 % MeCN/90 % HCOOH (0.01 %); 30 min: 60 % MeCN/40 % HCOOH (0.01 %) with a flow rate of 0.4 mL/min. UV spectra were recorded during HPLC analysis with a DAD instrument (Agilent).

MALDI-TOF analyses were performed on a Bruker Daltonics autoflex TOF/TOF mass spectrometer. The sponge powder (1 – 2 mg) was suspended in 50 μL of a 1 : 1 mixture of MeCN and TFA (2.5 % in water) and incubated for 10 min. 2 μL of the solution was placed on a ground steel target and dried. Afterwards 2 μL of matrix solution (HCCA) was placed on the target. The spectra were calibrated using ribosomal proteins from *E. coli* (DH5α). Due to a missing accurate calibration of the mass spectrometer in the low molecular range (*m/z* = 200 – 1000), the resulting *m/z* values could not be used for the generation of molecular formulae.

The applied mass range was 0 – 3000 Dalton for linear mode and 0 – 1000 Dalton for LIFT® spectra. Spectra obtained from 450 laser shots were combined. The acquired spectra were smoothed applying Savitzky Golay smoothing parameters (*m/z* = 0.1 and 1 cycles).

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