Structure of Potential Dithiolopyrrolone Antibiotics Detected from the DART-ToF-MS Spectra of Saccharothrix algeriensis Extract

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1 Introduction

Dithiolopyrrolones (DTPs) are members of the pyrrothine class of naturally occurring antibiotics that are characterised by the possession of 4H-[1,2]dithiolopyrrolo[4,3-b]pyrrol-5-one skeleton. Currently, there are more than 30 known natural DTP antibiotics. This class of potent antibiotics includes many compounds such as thiolutin, butyryl-pyrrothine, iso-butyryl-pyrrothine, senecioyl-pyrrothine/tigloyl-pyrrothine, valeryl-pyrrothine/iso-valeryl-pyrrothine, 2-methyl-3-pentenyl-pyrrothine/2-hexonyl-pyrrothine, iso-hexanoyl-pyrrothine and benzoyl-pyrrothine were characterised by their exact mass measurement and the corresponding molecular formula of each compound. The obtained results confirmed that DART-ToF-MS is an appropriate confirmatory technique for powerful and rapid screening, as well as characterisation of bacterial secondary metabolites.

Keywords
Saccharothrix algeriensis, direct analysis in real time, time-of-flight mass spectrometry, dithiolopyrrolone analogs, antibiotics

Dithiolopyrrolones (DTPs) have very broad biological activities against various bacteria and eukaryotic organisms. In addition, these molecules exhibit strong anti-cancer activity against several human cancer cell lines. Multiple mechanisms contribute to the antibacterial activity of DTP derivatives including the inhibition of RNA synthesis and the inhibition of the initiation, elongation or transcription steps of RNA synthesis. The structural characteristics of DTP core scaffold, as reported by Qin et al., may give some indication to the mode of action. The mycotoxin gliotoxin possesses a similar disulphide bond, and the reduction of the disulphide bond in the cell gives rise to the more active di-thiol groups which can react with target proteins’ thiol groups.

The bacterium Saccharothrix algeriensis produced five DTP antibiotics containing N-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolopyrrolo[4,3-b]pyrrole with different branched chains of acyl groups: thiolutine (also named acetyl-pyrrothine, aceto-pyrrothine or farcinicine), butyryl-pyrrothine (also named butyro-pyrrothine, butanoyl-pyrrothine or xenorhabdin VIII), iso-butyryl-pyrrothine (also named iso-butyro-pyrrothine or 2-methyl-propionyl-pyrrothine), senecioyl-pyrrothine (also named 3-methyl-2-butenoyl-pyrrothine), and tigloyl-pyrrothine (also named isovaleryl-pyrrothine). Seven other new DTP analogs: iso-valeryl-pyrrothine (h), 2-hex-
The actinobacterium *Saccharothrix algeriensis* NRRL B-24137 (= DSM 44581) was used throughout this study as reported by Bouras et al. It was grown and maintained at 4 °C on slants of ISP2 solid medium containing (per litre of distilled water): 4 g dextrose (D-glucose), 4 g malt extract, 4 g yeast extract, and 18 g agar. The pH of the medium was adjusted to 7.0 with a 2 M NaOH solution prior to autoclaving at 121 °C for 20 min.

2 Experimental

2.1 Producing actinobacterial strain

The actinobacterium *Saccharothrix algeriensis* NRRL B-24137 (= DSM 44581) was used throughout this study as reported by Bouras et al. It was grown and maintained at 4 °C on slants of ISP2 solid medium containing (per litre of distilled water): 4 g dextrose (D-glucose), 4 g malt extract, 4 g yeast extract, and 18 g agar. The pH of the medium was adjusted to 7.0 with a 2 M NaOH solution prior to autoclaving at 121 °C for 20 min.

2.2 Culture medium

A basal semi-synthetic (BSS) medium was used for both preculture and production of antibiotics as reported by Bouras et al. This medium consisted of (per litre of distilled water): 10 g dextrose, 2 g (NH₄)₂SO₄, 2 g NaCl, 0.5 g KH₂PO₄, 1 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 5 g CaCO₃, and 2 g yeast extract. The pH of the medium was adjusted to 7.0 using a 2 M NaOH solution before autoclaving. The dextrose was autoclaved separately to avoid the chemical reaction between nitrogen sources and reducing carbon sources that gives a brown colour (Millard reaction), and then added aseptically to the culture medium before inoculation.

2.3 Culture conditions

The DTP antibiotic production was investigated in the BSS medium. The preculture (250 ml Erlenmeyer flask containing 50 ml of the culture medium) was incubated for 48 h on a model G25 gyratory shaker (New Brunswick Scientific Co., New Jersey, USA) at 260 rpm and 30 °C. The preculture was then homogenised, and 5 ml was used to inoculate 100 ml of the same medium in 500-mm Erlenmeyer flask. The incubation temperature was kept at 30 °C throughout the 72 h fermentation period (in general, DTP antibiotic production reached a maximum at 72 h after inoculation).

2.4 Extraction of DTP antibiotics

The extraction of DTP antibiotics took place on the day of optimal production (after 3 days of fermentation). The culture broth was centrifuged for 20 min at 8000 ×g to remove the mycelium. The cell-free supernatant was extracted with an equal volume of dichloromethane. The organic layer was dehydrated with Na₂SO₄ and evaporated to dryness by a rotary evaporator (Laborota 4000) under a vacuum at 40 °C. The resulting dry extracts were recuperated in 1 ml of methanol and subjected to analysis.

2.5 DART-ToF-MS conditions

The high-resolution mass spectra were recorded on an AccuTOF LC-plus JMS-T100 LP mass spectrometer from JEOL (Tokyo, Japan). This instrument consisted of a DART ion source from Ion Sense (Saugus, MA, USA) operated under atmospheric pressure, and a high-resolution time-of-flight mass spectrometer, it produces a DART-ToF-MS system that is a powerful tool for characterising bioactive molecules. DART-ToF-MS has rapidly emerged as a powerful technique for profiling the major constituents of various kinds of samples without need of prior separation or pretreatment. Since this emerging technique was patented in the USA in 2005, it has been successfully applied in various fields such as pharmaceuticals, health sciences, food products analysis, quality control, explosives, material analyses, phytochemicals, synthetic and organic compounds, forensic sciences, pesticides, and environmental studies.

The goal of this work was to develop a rapid and accurate method for characterisation of DTP antibiotics (all characterised by the possession of N₂O₂S₂) in *S. algeriensis* directly from its intact dichloromethane organic layer using DART ion source coupled to ToF-MS.

ononyl-pyrothine (i), 2-methyl-3-pentenyl-pyrothine (j), iso-hexanoyl-pyrothine (k) (also named iso-pentyl-formyl-pyrothine), propionyl-pyrothine, crotonyl-pyrothine, and sorbyl-pyrothine were also obtained from *S. algeriensis* using the same technique. The structures of these compounds are shown in Fig. 1. However, many other DTP analogs were secreted in very small amounts remaining unknown (Bouras, unpublished data).

Direct analysis in real time (DART) ion source is a recently developed method, which allows ionization of most organic molecules under atmospheric pressure. It has proven to be an efficient and reliable technique, allowing for screening complex mixtures such as biochemical samples without sample preparation or chromatographic separation. When combined with a high-resolution mass analyser, such as time-of-flight mass spectrometer, it produces a DART-ToF-MS system that is a powerful tool for characterising bioactive molecules. DART-ToF-MS has rapidly emerged as a powerful technique for profiling the major constituents of various kinds of samples without need of prior separation or pretreatment. Since this emerging technique was patented in the USA in 2005, it has been successfully applied in various fields such as pharmaceuticals, health sciences, food products analysis, quality control, explosives, material analyses, phytochemicals, synthetic and organic compounds, forensic sciences, pesticides, and environmental studies.

The goal of this work was to develop a rapid and accurate method for characterisation of DTP antibiotics (all characterised by the possession of N₂O₂S₂) in *S. algeriensis* directly from its intact dichloromethane organic layer using DART ion source coupled to ToF-MS.
Table 1 – Determination of the accurate molecular weight of dithiopyrrolone antibiotics in S. algeriensis extract by DART-MS in positive ionisation mode (helium temperature: 250 °C, peak voltage: 500 V)

| Experimental mass / m/z | Calculated mass / m/z | Mass difference / mmu | Molecular formula | Unsaturation degree | Antibiotic                      |
|-------------------------|-----------------------|-----------------------|-------------------|---------------------|---------------------------------|
| 228.00594               | 228.00272             | 3.23                  | C₈H₆N₂O₂S₂        | 6.0                 | Thiolutin                       |
| 229.01218               | 229.01054             | 1.64                  | C₈H₆N₂O₂S₂        | 5.5                 |                                 |
| 230.01227               | 230.01390             | -1.63                 | C₈H₆N₂O₂S₂        | 5.5                 |                                 |
| 231.00995               | 231.00634             | 3.61                  | C₈H₆N₂O₃S⁺        | 5.5                 |                                 |
| 256.03876               | 256.03402             | 4.74                  | C₁₀H₈N₃O₂S₂       | 6.0                 | Butyryl-pyrothine               |
| 257.04757               | 257.04184             | 5.72                  | C₁₀H₈N₃O₂S₂       | 5.5                 | Iso-butyryl-pyrothine           |
| 268.03510               | 268.03402             | 1.09                  | C₁₀H₈N₃O₂S₂       | 7.0                 | Senecioyl-pyrothine             |
| 269.04328               | 269.04184             | 1.43                  | C₁₀H₈N₃O₂S₂       | 6.5                 | Tigloyl-pyrothine               |
| 270.04467               | 270.04967             | -5.00                 | C₁₀H₈N₃O₂S₂       | 6.0                 | Valeryl-pyrothine               |
| 271.04936               | 271.05749             | -8.13                 | C₁₀H₈N₃O₂S₂       | 5.5                 | Iso-valeryl-pyrothine           |
| 282.04865               | 282.04967             | -1.02                 | C₁₀H₈N₃O₂S₂       | 7.0                 | 2-Hexonyl-pyrothine             |
| 283.06317               | 283.05749             | 5.68                  | C₁₀H₈N₃O₂S₂       | 6.5                 | 2-Methyl-3-pentenyl-pyrothine   |
| 284.06876               | 284.06532             | 3.44                  | C₁₀H₈N₃O₂S₂       | 6.0                 | Iso-hexanoyl-pyrothine          |
| 290.02455               | 290.01837             | 6.18                  | C₁₀H₈N₃O₂S₂       | 10.0                | Benzoyl-pyrothine               |
| 291.03110               | 291.02619             | 4.91                  | C₁₀H₈N₃O₂S₂       | 9.5                 |                                 |
iso-hexanoyl-pyrothine (m/z 284), and benzoyl-pyrothine (m/z 290). All the characterised eleven DTPs were already reported to be produced by *S. algeriensis*.  

It was established that the positive ionisation in DART ion source can occur through three possible mechanisms by interaction with the heated and excited metastable helium atoms, with no or little fragmentation. Generally, the main observed peak corresponds to a protonated adduct ion [M+H]+; it results from protonation by interaction with atmospheric water molecules. A second possible mechanism can occur with highly unsaturated compounds by loss of an electron and formation of a radical molecular ion M+. The third ionisation process is less likely, it corresponds to the loss of hydride which leads to an [M-H]- ion. Indeed, the detected DTPs showed mainly the protonated molecular ion [M+H]+, beside the lower ion-radical M+.  

Fig. 3 shows enlarged portions of the high-resolution mass spectrum of the dichloromethane extract of *S. algeriensis*. The most intense peak at m/z 229.01070 corresponds to the protonated molecular ion of thiolulin with the molecular formula C10H8N2O2 and 5.5 as unsaturation degree. In the same peak cluster, the peak at m/z 228.00005 shows the same molecular formula as thiolulin C10H8N2O2; it is due to its molecular ion-radical. Due to the high intensity of these two molecular peaks of thiolulin, the contribution of minor isotope in carbon and sulphur can also be observed at m/z 230.01227 and 231.00995. They correspond to the protonated molecular ion of thiolulin [M+H]+ including either a carbon 13 or a sulphur 34 isotope, respectively. Another intense peak at m/z 269.04328 corresponds to the protonated molecular ion of senecioyl-pyrothine (tigloyl-pyrothine). Such as for thiolulin, the ion-radical molecular species is also present at m/z 268.03510 with the formula C11H13N2O2S2. However, due to overlapping with other DTP clusters, the minor peaks could not be observed. Similarly, the other dithiolopyrrolones characterised in Fig. 3 showed the presence of one or two molecular peaks M+ and [M+H]+ corresponding to butyryl-pyrothine, valeryl-pyrothine, 2-hexanoyl-pyrothine and benzoyl-pyrothine. When the ion intensity is low, the small peaks, due to the minor isotopes, are masked by the background signals.

On the other hand, it should be noted that some DTPs such as butyryl-pyrothine/iso-butyryl-pyrothine, and senecioyl-pyrothine/tigloyl-pyrothine, etc., have exactly the same molecular formula and exact mass m/z 256 and 268, respectively. Since DART ion source is a "soft" ionisation technique, which produces essentially the protonated molecular ion of each species with no or little fragmentation, it cannot differentiate between isomers that will correspond to the same peak in the spectrum. Therefore, they cannot be distinguished on the basis of high-resolution mass spectrometry such as DART-ToF-MS technique. However, all these isomers have been reported to be produced by *S. algeriensis*.  

As reported previously, some DTPs were detected by HPLC only after addition of some precursors to enhance their production. However, it is important to mention that, in the present work, all the detected DTPs were easily observed by DART-ToF-MS, without precursor feeding.

4 Conclusion  

The dichloromethane organic layer of *S. algeriensis* was investigated by DART-ToF-MS in positive ionisation mode. The interpretation of the high-resolution mass spectrum allowed determination of the accurate molecular weight and the possible formula of its main organic constituents. Among them, eleven DTP derivatives were characterised in the *S. algeriensis* extract without separation or sample treatment (except extraction). To the best of our knowl-
edge, antibiotics have never been studied by DART-ToF-MS, and this is the first application of this technique for characterisation of antibiotics, which resulted in confirmation of eleven DTPs.

**List of abbreviations**

| Abbreviation | Description                        |
|--------------|------------------------------------|
| DTP          | dithiolopyrrolone                  |
| DART-ToF-MS  | direct analysis in real time-time of flight-mass spectrometry |
| S. algeriensis | Saccharothrix algeriensis         |
| RNA          | ribonucleic acid                   |
| NRRL         | Northern Regional Research Laboratory |
| DSM          | Deutsche Sammlung von Mikroorganismen |
| ISP2         | International Streptomyces Project 2 |
| mDa          | milli Dalton                       |
| mmu          | milli mass unit                    |

**DECLARATION OF INTEREST STATEMENT**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SAŽETAK

Struktura potencijalnih ditiolopirolonskih antibiotika detektirana iz DART-ToF-MS spektra ekstrakta kulture Saccharothrix algeriensis

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Ditiolopirolonski antibiotici koje proizvodi saharska micelijska bakterija Saccharothrix algeriensis poznati su po svom snažnom biološkom djelovanju. Biokemijsko profiliranje ekstrakta kulture S. algeriensis učinjeno je direktnom analizom u realnom vremenu uz masenu spektrometriju vremena leta (DART-ToF-MS). Nije objavljena nijedna druga studija na ditiolopirolonima koja primjenjuje tu tehniku. Pronađeno je jedanaest derivata ditiolopirolona: tiolutin, butiril-pirotin/izo-butiril-pirotin, senecioil-pirotin/tigloil-pirotin, valeril-pirotin/izo-valeril-pirotin, 2-metil-3-pentenil-pirotin/2-heksonil-pirotin, izo-heksanoil-pirotin i benzoil-pirotin. Dobiveni rezultati potvrdili su da je DART-ToF-MS prikladna tehnika za moćan i brzi “screening”, kao i za karakterizaciju sekundarnih metabolita bakterija.

Ključne riječi
Saccharothrix algeriensis, direktna analiza u realnom vremenu, ToF-MS spektroskopija, analozi ditiolopirolona, antibiotici

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