https://doi.org/10.15255/KUI.2021.083
KUI-40/2022
Original scientific paper
Received December 15, 2021
Accepted February 18, 2022

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

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Abstract

Dithiolopyrrolone antibiotics, produced by the Saharan mycelial bacterium *Saccharothrix algeriensis*, are known for their potent biological activities. Biochemical profiling of *S. algeriensis* culture extract was done by direct analysis in real time and time-of-flight mass spectrometry (DART-ToF-MS). No other study on dithiolopyrrolones by this technique has been published. Eleven dithiolopyrrolone derivatives: 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

1 Introduction

Dithiolopyrrolones (DTPs) are members of the pyrrothine class of naturally occurring antibiotics that are characterised by the possession of 4*H*-[1,2]dithiolo[4,3-*b*]pyrrol-5-one skeleton.[1] 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, holomycin, holothin, propionyl-pyrrothine (also named propio-pyrrothine or aureothricin), propionyl-holothin, xenorhabdins, xenorxides, and thiomarinols.[2–7] DTP derivatives were obtained from certain strains and species of *Streptomyces*,[2–5] *Xenorhabdus*,[4] *Pseudoalteromonas*,[6–8] *Photobacterium*,[9] *Yersinia*,[10] and from the Saharan mycelial bacterium *Saccharothrix algeriensis* NRRL B-24137.[3–11]

DTPs have very broad biological activities against various bacteria and eukaryotic organisms.[3,7,12–16] In addition, these molecules exhibit strong anti-cancer activity against several human cancer cell lines.[7,17,18] Multiple mechanisms contribute to the antibacterial activity of DTP derivatives including the inhibition of RNA synthesis[19] and the inhibition of the initiation, elongation or transcription steps of RNA synthesis.[20]

The structural characteristics of DTP core scaffold, as reported by *Qin et al.*,[10] a disulphide-bridged heterocycle[10] 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 dithiol groups which can react with target proteins' thiol groups.[21–22]

The bacterium *S. algeriensis* produced five DTP antibiotics containing *N*-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo[4,3-*b*]pyrrole with different branched chains of acyl groups: thiolutine (**a**) (also named acetyl-pyrrothine, aceto-pyrrothine or farcinicine), butyryl-pyrrothine (**b**) (also named butyro-pyrrotine, butanoyl-pyrrothine or xenorhabdin VII), iso-butyryl-pyrrothine (**c**) (also named iso-butyro-pyrrothine or 2-methyl-propanoyl-pyrrothine), senecioyl-pyrrothine (**d**) (also named 3-methyl-2-butenoyl-pyrrothine), and tigloyl-pyrrothine (**e**), as described by *Lamari et al.*[3] and *Bouras et al.*[23] Furthermore, *Bouras et al.*[1] used a precursor directed biosynthesis approach (addition of direct precursor) to generate new DTP derivatives: benzoyl-pyrrothine (**f**) and valeryl-pyrrothine (**g**) (also named pentanoyl-pyrrothine).[1] Seven other new DTP analogs: iso-valerylpyrrothine (**h**), 2-hex-

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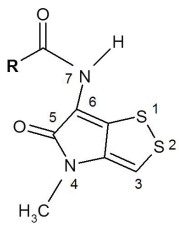

R:	CH_3	thiolutin (a)
R:	$(CH_2)_2$-CH_3	butyryl-pyrrothine (b)
R:	$CH(CH_3)_2$	iso-butyryl-pyrrothine (c)
R:	$CH=C(CH_3)_2$	senecioyl-pyrrothine (d)
R:	$C(CH_3)=CH(CH_3)$	tigloyl-pyrrothine (e)
R:	C_6H_5	benzoyl-pyrrothine (f)
R:	$(CH_2)_3$-CH_3	valeryl-pyrrothine (g)
R:	$CH(CH_3)(CH_2CH_3)$	iso-valeryl-pyrrothine (h)
R:	$CH=CH$-$CH_2CH_2CH_3$	2-hexonyl-pyrrothine (i)
R:	$CH=C(CH_3)(CH_2CH_3)$	2-methyl-3-pentenyl-pyrrothine (j)
R:	$(CH_2)_2$-$CH(CH_3)_2$	iso-hexonyl-pyrrothine (k)

Fig. 1 – Structure of DTP antibiotics detected from the DART mass spectra of *S. algeriensis* extract

onyl-pyrrothine (**i**), 2-methyl-3-pentenyl-pyrrothine (**j**), iso-hexanoyl-pyrrothine (**k**) (also named iso-pentyl-formyl-pyrrothine), propionyl-pyrrothine, crotonyl-pyrrothine, and sorbyl-pyrrothine were also obtained from *S. algeriensis* using the same technique.[24–27] 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.[28–30] 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.[31] Since this emerging technique was patented in the USA in 2005[32,33], 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.[34–37]

The goal of this work was to develop a rapid and accurate method for characterisation of DTP antibiotics (all characterised by the possession of $N_2O_2S_2$) in *S. algeriensis* directly from its intact dichloromethane organic layer using DART ion source coupled to ToF-MS.

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.*[11] 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.*[23] This medium consisted of (*per* litre of distilled water): 10 g dextrose, 2 g $(NH_4)_2SO_4$, 2 g NaCl, 0.5 g KH_2PO_4, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCO_3$, 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 Na_2SO_4 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 analyser. All the obtained mass spectra were acquired using positive ionisation mode. The main parameters of DART ion source and ToF mass spectrometer were investigated and optimised in order to obtain the best mass resolution and signal intensity for the studied samples. The selected experimental conditions are given in detail further herein. The samples were vaporised and ionised using helium at a flow-rate of 4 l min^{-1} and heated at 250 °C. The discharge needle electrode of the DART ionisation source was set at a 3.0 kV potential, while the perforated and grid electrodes were set at 100 and 250 V voltages, respectively. For sample introduction using a glass rod, the gap distance separating the ionisation source outlet and the mass spectrometer inlet was 20 mm. For ion transfer from the DART source to the mass analyser, the potentials of orifice 1, orifice 2, and ring lens were set at 20, 5, and 13 V, respectively, and the potential of the radio-frequency guide was 500 V. The accurate mass spectra were acquired in the *m/z* range between 100 and 500 Da using a recording interval of 1 s. For mass drift compensation and accurate mass determination, a solution of polyethylene glycol (PEG 200) in methanol (200 μg ml^{-1}) was used as calibration standard and injected before each sample. Data acquisition and processing were performed using the MassCenter (version 1.3) software from JEOL. In the investigated range, the mass spectra were obtained with a mass resolution between 3400 and 3900.

3 Results and discussion

3.1 Characterisation of the compounds in the mass spectra

Mass spectrometry is one of the most powerful analytical methods available for determining the structure of microbial (bacterial and fungal) secondary metabolites. This powerful technique could be very useful for investigation of bacterial secondary metabolites. DART-ToF-MS has been used for the rapid screening of various natural complex samples, but its use in the characterisation of DTP antibiotics has not been reported previously.

In this study, the high-resolution mass spectrum of the dichloromethane organic layer of *S. algeriensis* was recorded using DART-ToF-MS. The accurate molecular weight of the main constituents was determined and their formula deduced, allowing the characterisation of several DTPs. To confirm the results, the contribution of the minor isotopes of carbon and sulphur was also checked to establish the molecular formula of each DTP (all characterised by the possession of $N_2O_2S_2$).

Fig. 2 shows the high-resolution mass spectrum of *S. algeriensis* dichloromethane organic layer in positive ionisation mode. The peaks corresponding to the main constituents of the investigated extract were observed in the mass range *m/z* 100 to 500 Da, and after calibration and processing of the experimental results, the accurate molecular weights were obtained with five decimals. The main results obtained from interpretation of this spectrum are reported in Table 1; they show the experimental (measured) mass, the calculated mass, the mass difference (in mmu or mDa), and the proposed molecular formula for each indexed peak. Also, the unsaturated degree of each compound helps to predict the chemical structure of the expected antibiotic. The peaks corresponding to DTP species in dichloromethane organic layer were assigned to: thiolutin (*m/z* 228), butyryl-pyrrothine/iso-butyryl-pyrrothine (*m/z* 256), senecioyl-pyrrothine/tigloyl-pyrrothine (*m/z* 268), valeryl-pyrrothine/iso-valeryl-pyrrothine (*m/z* 270), 2-hexonyl-pyrrothine/2-methyl-3-pentenyl-pyrrothine (*m/z* 282),

Table 1 – Determination of the accurate molecular weight of dithiolopyrrolone 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	$C_8H_8N_2O_2{}^{32}S_2$	6.0	Thiolutin
229.01218	229.01054	1.64	$C_8H_9N_2O_2{}^{32}S_2$	5.5	
230.01227	230.01390	−1.63	$C_7{}^{13}CH_9N_2O_2{}^{32}S_2$	5.5	
231.00995	231.00634	3.61	$C_8H_9N_2O_2{}^{32}S^{34}S$	5.5	
256.03876	256.03402	4.74	$C_{10}H_{12}N_2O_2{}^{32}S_2$	6.0	Butyryl-pyrrothine Iso-butyryl-pyrrothine
257.04757	257.04184	5.72	$C_{10}H_{13}N_2O_2{}^{32}S_2$	5.5	
268.03510	268.03402	1.09	$C_{11}H_{12}N_2O_2{}^{32}S_2$	7.0	Senecioyl-pyrrothine Tigloyl-pyrrothine
269.04328	269.04184	1.43	$C_{11}H_{13}N_2O_2{}^{32}S_2$	6.5	
270.04467	270.04967	−5.00	$C_{11}H_{14}N_2O_2{}^{32}S_2$	6.0	Valeryl-pyrrothine Iso-valeryl-pyrrothine
271.04936	271.05749	−8.13	$C_{11}H_{15}N_2O_2{}^{32}S_2$	5.5	
282.04865	282.04967	−1.02	$C_{12}H_{14}N_2O_2{}^{32}S_2$	7.0	2-Hexonyl-pyrrothine 2-Methyl-3-pentenyl-pyrrothine
283.06317	283.05749	5.68	$C_{12}H_{15}N_2O_2{}^{32}S_2$	6.5	
284.06876	284.06532	3.44	$C_{12}H_{16}N_2O_2{}^{32}S_2$	6.0	Iso-hexanoyl-pyrrothine
290.02455	290.01837	6.18	$C_{13}H_{10}N_2O_2{}^{32}S_2$	10.0	Benzoyl-pyrrothine
291.03110	291.02619	4.91	$C_{13}H_{11}N_2O_2{}^{32}S_2$	9.5	

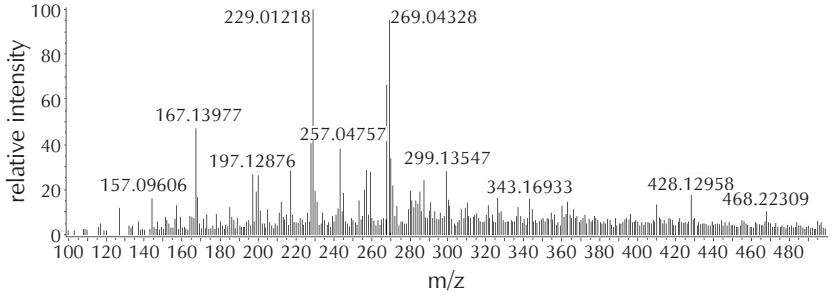

Fig. 2 – DART-ToF-MS high-resolution mass spectrum of the dichloromethane organic layer of *S. algeriensis*. Conditions used: positive ionisation, peak voltage: 500 V, helium temperature: 250 °C.

iso-hexanoyl-pyrrothine (*m/z* 284), and benzoyl-pyrrothine (*m/z* 290). All the characterised eleven DTPs were already reported to be produced by *S. algeriensis*.[1,3,24–27] 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 a 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 $M^{+\cdot}$.

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 thiolutin with the molecular formula $C_8H_9N_2O_2S_2$ and 5.5 as unsaturation degree. In the same peak cluster, the peak at *m/z* 228.00005 shows the same molecular formula as thiolutin $C_8H_8N_2O_2S_2$; it is due to its molecular ion-radical. Due to the high intensity of these two molecular peaks of thiolutin, the contribution of minor isotopes in carbon and sulphur can also be observed at *m/z* 230.01227 and 231.00995. They correspond to the protonated molecular ion of thiolutin $[M+H]^+$ 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-pyrrothine (tigloyl-pyrrothine). Such as for thiolutin, the ion-radical molecular species is also present at *m/z* 268.03510 with the formula $C_{11}H_{12}N_2O_2S_2$. 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-pyrrothine, valeryl-pyrrothine, 2-hexonyl-pyrrothine and benzoyl-pyrrothine. 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-pyrrothine/iso-butyryl-pyrrothine, and senecioyl-pyrrothine/tigloyl-pyrrothine, *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*.[1,24–27]

As reported previously, some DTPs were detected by HPLC only after addition of some precursors to enhance their production.[1,24–27] 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.

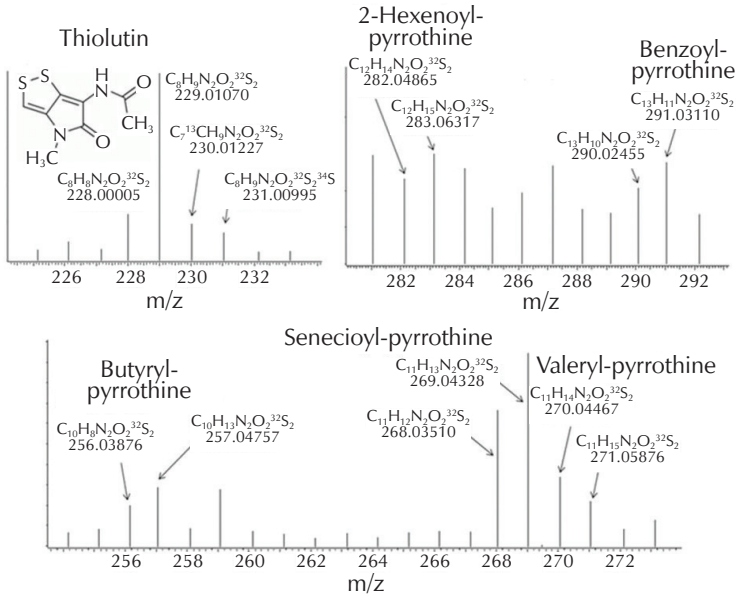

Fig. 3 – Main peaks of the DTPs characterised by DART-ToF-MS in *S. algeriensis*. Conditions used: positive ionisation, peak voltage: 500 V, helium temperature: 250 °C.

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

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 bakterije *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-pentenelin-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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Izvorni znanstveni rad
Prispjelo 15. prosinca 2021.
Prihvaćeno 18. veljače 2022.