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Short communication

On-line identification of 4″-isovalerylspiramycin I in the genetic engineered strain of *S. spiramyceticus* F21 by liquid chromatography with electrospray ionization tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry

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ABSTRACT

LC-hyphenated techniques were applied to the on-line identification of isovalerylspiramycin I (isp I), a spiramycin-like macrolide in the crude extract of fermentation broth from a genetically engineered strain of *S. spiramyceticus* F21. In the structural characterization of the large molecular secondary metabolite of isp I, LC–DAD-UV–ESI-MS^n analysis played a crucial role, and stop-flow LC–^{1}H NMR measurement, with bitespiramycin used as reference, was a valuable complement approach. This rational approach proved to be an efficient means for the rapid and accurate structural determination of known microbial secondary metabolites, by which targeted isolation of component(s) of interest can be subsequently performed for further biological and pharmacological studies in drug development.

1. Introduction

Bitespiramycin (BT), which is produced by *S. spiramyceticus* F21 harboring a 4″-*O*-acyltransferase gene from *S. mycarofaciens* 1748, is a hybrid 16-membered macrolide antibiotic developed in a new drug discovery program in China for antibacterial infections [1]. It showed excellent therapeutic effects in both phase II and III clinical trials.

BT, a multi-component antibiotic [2], is a derivative from spiramycin (SP) which consists of three components: SP I, II, and III. For practical purposes, there is little distinction between these SP components. To simplify the multi-component character of BT, a new genetically engineered strain, WSJ-2, was constructed by inactivating the gene encoding the 3-*O*-acyltransferase for the acylation of SP I (3-OH) in the BT producing strain.

It is advisable to recognize the microbial secondary metabolites at the earliest stage of the construction of the genetically engineered strain as possible. LC coupled techniques can provide both efficient separation of metabolites and valuable spectroscopic information about the constituents at the same time, using only several micrograms of sample [3–5]. At present, mass spectrometry (MS) is one of the most sensitive and selective methods of molecular analysis. HPLC coupled to MS (LC–MS) can provide information about the molecular weight as well as the structure of the analytes thanks to the multi-stage tandem MS (LC–MS^n) [6]. HPLC coupled with nuclear magnetic resonance (LC–NMR) plays a key role in providing detailed structural information about organic compounds in solution [7,8]. Nevertheless, LC–NMR has mostly been applied to the study of metabolic pathways or degradation products [9–11], and the applications to microbial secondary metabolites isolation or purification have scarcely been reported [12]. Of course, the inherent drawbacks of this technique, such as lack of sensitivity, loss of significant parts of proton spectra through solvent suppression [13,14] and differences in chemical shifts between standard and on-line spectra [15], might hinder its use in the structural identification of natural products. The difficulties of dealing with microbial secondary metabolites include its intrinsic molecular diversity, constituent multiplicity and low concentration(s) of interested composition(s), that make it a challenging task for on-line separation and direct NMR detection on crude extract.

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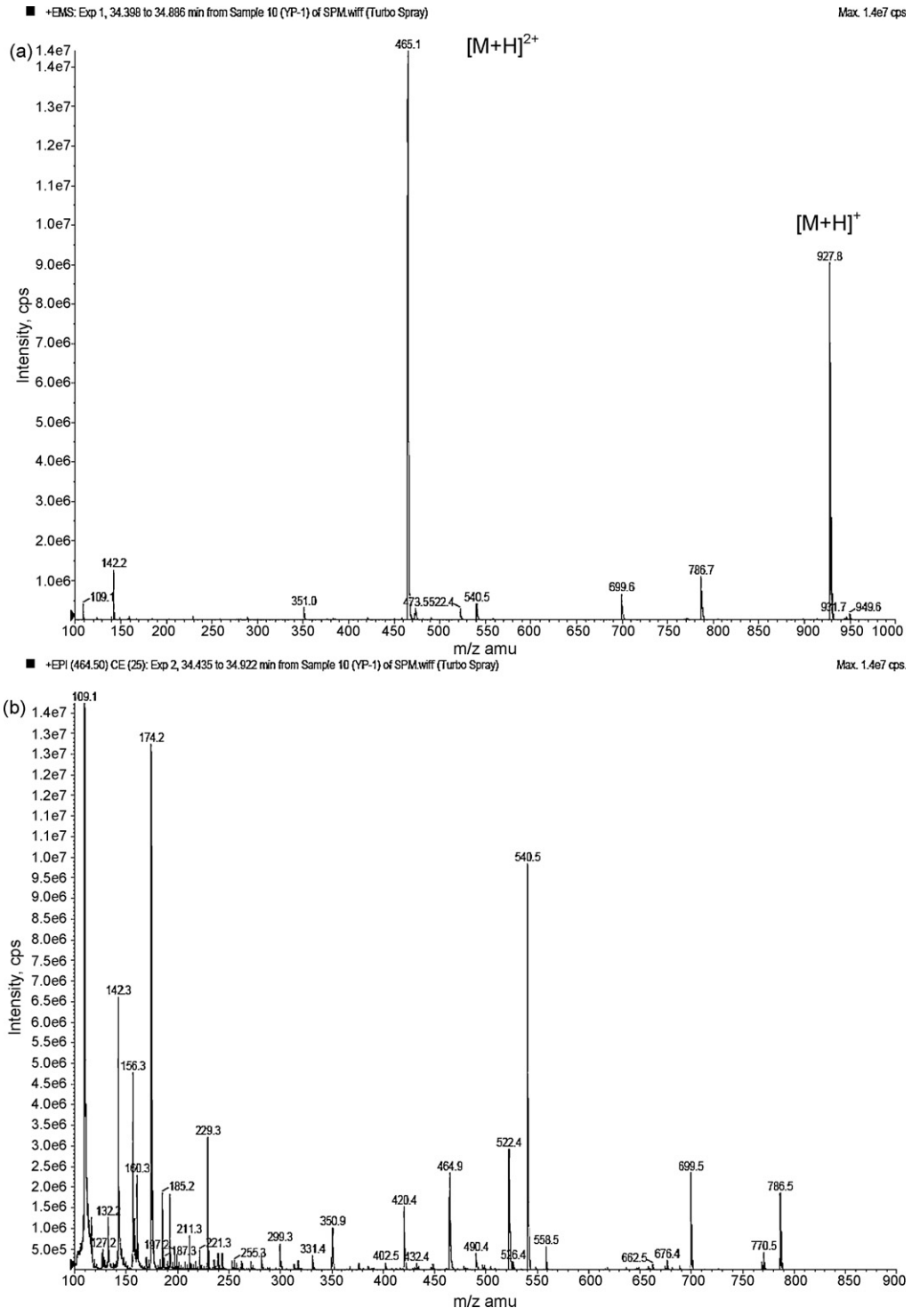

Fig. 1. LC–ESI-MS–MS spectra recorded on-line for constituent 1 in the enriched fraction. (a) MS^1 spectrum. (b) MS^2 spectrum.

In this paper, we report an on-line identification of a spiramycin derivative produced from WSJ-2 by means of a combination of the different LC coupled techniques mentioned above. The method presented here took advantage of the combined application of LC–DAD-UV, LC–ESI-MS–MS and LC–^{1}H NMR to the crude fermentation extract. This allowed a rapid structural characterization of the main constituent in the secondary metabolites of the genetically engineered WSJ-2 as 4″-isovalerylspiramycin I (isp I).

2. Experimental

2.1. Materials

The fermentation broth of WSJ-2 was extracted with ethyl acetate (EtOAc). The crude extract was partitioned by countercurrent chromatography using petroleum ether–ethyl acetate–methanol–water (1:3:2:2) that led to an enriched fraction of the interested constituent 1 (isp I).

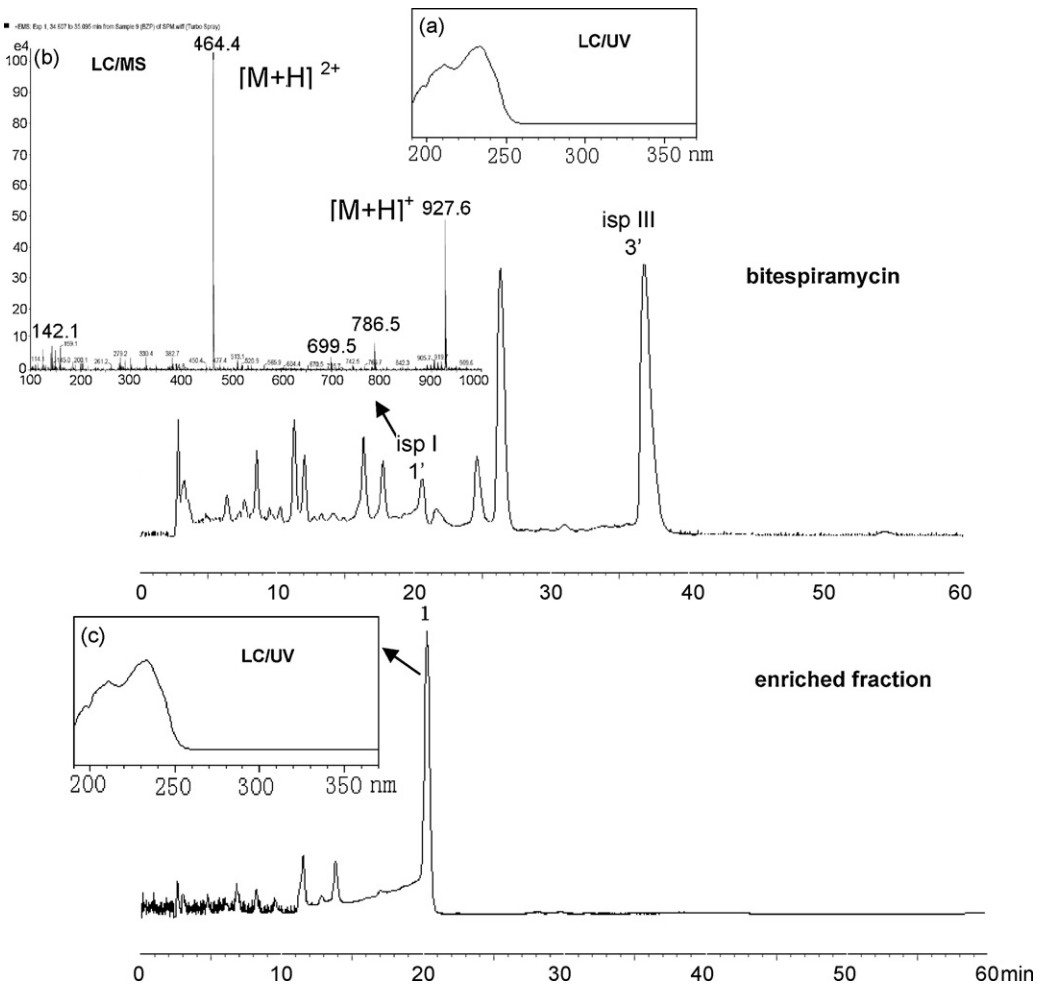

Fig. 2. LC–DAD-UV–ESI-MS^n analyses of the enriched fraction and BT using a mobile phase of MeCN:10 mM (53:47) with UV detection at 232 nm. Insert: (a) UV spectrum of peak 1′ corresponding to isp I in BT; (b) LC–ESI-MS spectrum of peak 1′ in BT; (c) UV spectrum of constituent 1 in the enriched fraction.

BT, which contained isp I as one of its components [2], was used in this study as reference. It was extracted and purified from the fermentation broth of *S. spiramyceticus* F21 in our lab [16]. Its component structures had been reported as determined on the basis of off-line IR, UV, MS, 1D and 2D NMR spectral analyses [17–20].

2.2. Solvents and reagents

Acetonitrile (MeCN) was HPLC-grade (Fisher, Fair Lawn, USA). Water (Watsons, Guangzhou, China) was double-distilled. Deuterated water (isotopic purity 99.8 atom% D) was obtained from Merck KGaA (Darmstadt, Germany). Both solvents were passed through Millipore filters (water solution: 0.45 μm HA; MeCN: 0.50 μm FH) (Bedford, MA, USA). Ammonium acetate for spectroscopy was purchased from Beijing Chemical Reagents Company (Beijing, China).

2.3. LC–DAD-UV analysis

Reversed-phase HPLC of the crude EtOAc extract was performed on a LC-10ATvp (Shimadzu, Japan) photodiode array detection (DAD) liquid chromatography system. The separation was achieved on a Kromasil C_{18} prepacked column (Dalian, Liaoning, China) (150 mm × 4.6 mm i.d.; 5 μm) with MeCN–10 mM ammonium acetate gradient elution (10:90 to 100:0; 90 min). The flow-rate was 1 ml/min; the UV traces were measured at 232 nm and UV spectra (DAD) were recorded between 190 and 370 nm on-line.

2.4. LC–ESI-MS^n analyses

For the on-line LC–MS^n analyses, an Agilent 1100 Series liquid chromatography system was utilized, which was coupled to the QTRAP LC–MS–MS spectrometer from Applied Biosystems/MDS SCIEX (Condord, Ont, Canada) equipped with a Turbo Ionspray ion source. Chromatographic separation was carried out on a YMC C_{18} prepacked column (YMC, Japan) (150 mm × 4.6 mm i.d.; 5 μm) with MeCN–10 mM ammonium acetate (53:47) as the mobile phase. The electrospray ionization (ESI) conditions were: curtain gas, 12 units; collision gas pressure, high; spray voltage, 5.5 kV; nebulizer gas, 40 units; auxiliary gas, 30 units; turbogas temperature, 300 °C; declustering potential (DP), 80 V. Nitrogen was used in all cases. The collision energy was 25 eV.

2.5. LC–^{1}H NMR analyses

A Varian INOVA-500 spectrometer equipped with ^{1}H[^{13}C] pulsed field gradient LC–NMR flow probe with a 60 μL flow cell was used. Reversed-phase HPLC was carried out on a Varian (Palo Alto, CA, USA) modular HPLC system consisting of a Varian Prostar 230 solvent delivery system and Varian Prostar 330 photodiode array detector. The separation was performed under the same conditions as for the LC–ESI-MS^n analyses except that the water of the LC elution system was replaced by deuterated water (D_2O). An injection of 25 μL (3 mg/100 μL for enriched fraction, 5 mg/100 μL for BT) was made, which achieved a satisfactory LC–NMR detection. ^{1}H NMR

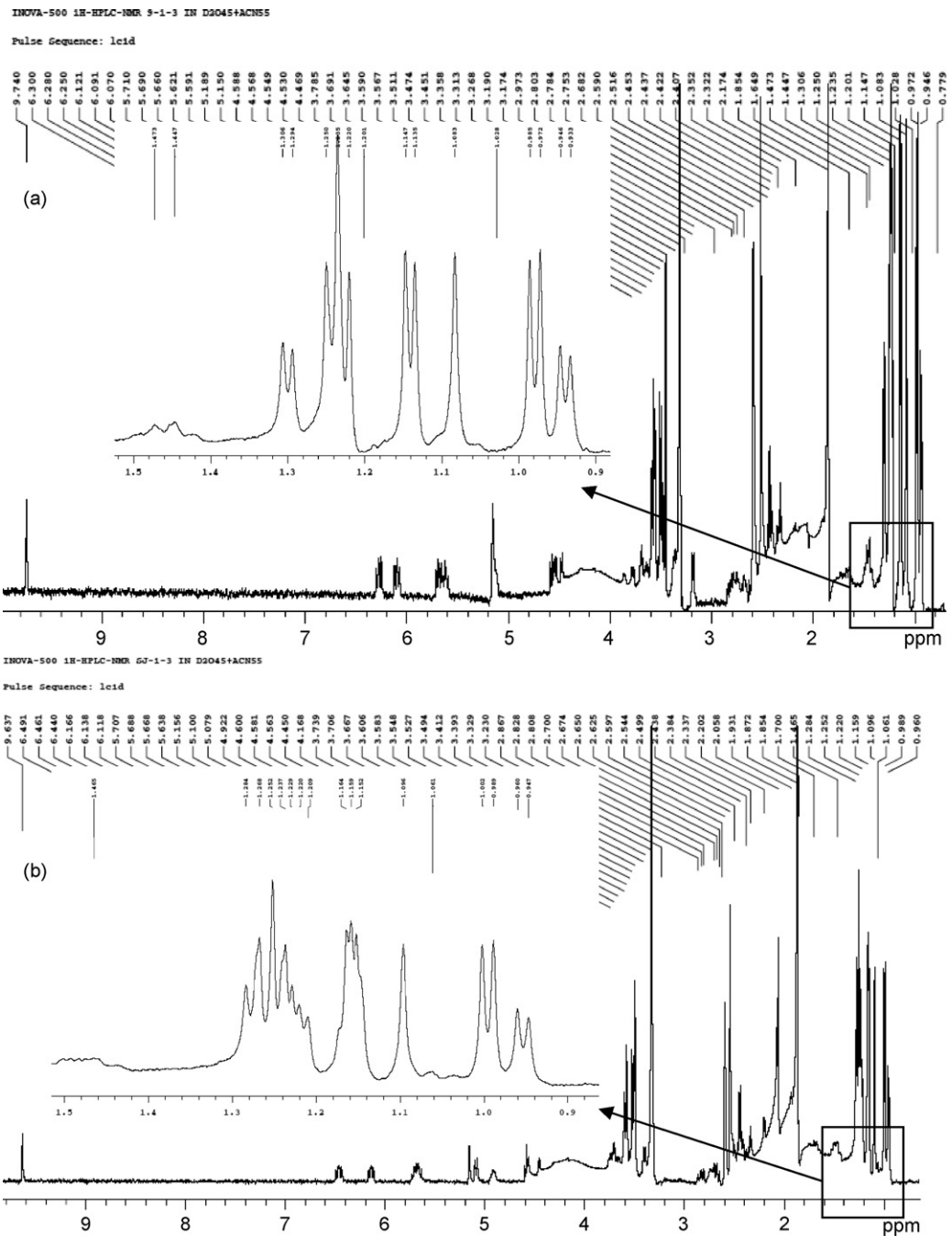

Fig. 3. LC–^{1}H NMR spectra of constituent 1 (a) and peak 3′ corresponding to isp III in BT (b).

measurements were recorded in stop-flow mode in order to obtain good quality LC–^{1}H NMR spectra. TMS was used as internal standard. Solvent suppression was performed on-line by the use of the WET sequence. A total of 256 transients were acquired to obtain the ^{1}H NMR data.

3. Results and discussion

3.1. Preliminary LC–UV and LC–ESI-MS–MS analyses

In order to obtain preliminary information concerning the composition of the crude extract, a combined LC–DAD-UV and positive ion LC–ESI-MS–MS analysis was carried out by reversed-phase HPLC with a broad acetonitrile–water gradient. The LC–UV chromatogram revealed six main peaks (data are not shown) with a major peak of constitute 1 at the retention time of 50.7 min. The separated components shared a similar type of UV spectrum with one maximal absorption band recorded at 232 nm (Fig. 2c), which suggested the existence of a typical α,β,γ,δ-unsaturated ether or alcohol chromophore of spiramycin-like macrolides.

The LC–ESI-MS spectrum of constituent 1 in the crude extract of WSJ-2 exhibited a protonated ion at *m*/*z* 927 $[M+H]^+$, indicating a molecular weight of 926 Da (Fig. 1a). Furthermore, the characteristic fragments of spiramycin could be clearly observed at *m*/*z* 142 for forosamine, *m*/*z* 174 for mycaminose and *m*/*z* 229 for isovalerylmycarose unit, respectively, which were presented in the LC–ESI-MS–MS spectrum (Fig. 1b). And, the corresponding macrolide residues due to sugar losses were also found at *m*/*z* 786,

699, 540 in the on-line mass spectra, respectively. All these diagnostic ions related to spiramycin or isp I by means of LC–MS have been reported previously [21–23]. Particularly, a dehydrated fragment displayed at *m*/*z* 522 indicated a hydroxyl on the macrolide ring and a rather weak ion at *m*/*z* 490 owning to a loss of 32 Da following dehydration suggested a methoxyl group on the macrolide ring. In the same manner, the LC–DAD-UV–ESI-MS^n spectra of BT provided efficient location of the LC-peak 1′ associated with isp I (Fig. 2).

With these structurally important ions and the characteristic UV spectrum data, it can be deduced that constituent 1 was isovaleryl-spiramycin I as in BT.

3.2. Stop-flow LC–^{1}H NMR analyses

In order to obtain more structural information about constituent 1 and to ascertain its structure on-line, a stop-flow LC–^{1}H NMR analysis was carried out on the enriched fraction. The quantity of the fraction injected onto the column was significantly increased to 750 μg without further separating the peaks. Two doublets at δ 4.47 (1H, J = 8.0 Hz) and 4.54 (1H, J = 9.5 Hz) were attributed to the anomeric H-1′ of mycaminose unit and H-1‴ of forosamine unit, respectively (Fig. 3a). The signal of another anomeric H-1″ in isovalerylmycarose moiety recorded at δ 5.15 (1H, d) overlapped a methine group H-15 (m) resonance. The presences of two methyl groups linked to a methine group in isovalerylmycarose unit were confirmed by a doublet at δ 0.98 (6H, J = 6.5 Hz) in the LC–^{1}H NMR spectrum. The other six methyl groups in the molecule were observed between δ 0.93 and 1.31: the singlet at δ 1.08 (3H) was assigned to CH_3-7″ in isovalerylmycarose unit; the doublet at δ 1.14 (6H, J = 6.0 Hz) suggested CH_3-6″ and CH_3-6‴ of isovalerylmycarose unit and forosamine unit, respectively; two doublets at δ 0.93 (3H, J = 6.5 Hz) and δ 1.30 (3H, J = 6.0 Hz) were assigned to CH_3-19 and CH_3-16, respectively, both substituted on the macrolide ring; the doublet corresponding to CH_3-6′, however, was shielded by a triplet at δ 1.2 attributable to solvent impurities. Furthermore, two strong signals of methyl groups linked to the nitrogen atom were observed at δ 2.51 (S, 6H) and 2.63 (S, 6H), owing to protons *N*–$(CH_3)_2$–7‴8‴ and *N*–$(CH_3)_2$–7′8′, respectively. The presence of the methoxyl group was confirmed by a singlet at δ 3.45 (3H) as deduced from the LC–ESI-MS–MS spectrum. A doublet at δ 3.77 (1H, J = 11.0 Hz) was attributed to a methine proton in position 3, which was substituted by a hydroxyl. The LC–^{1}H NMR spectrum that showed signals in range from δ 5.5 to 6.4 were characteristic of four olefinic protons for H-10 at δ 5.66 (1H, dd, J = 15.0,10.0 Hz), H-11 at δ 6.25 (1H, dd, J = 15.0, 11.0 Hz), H-12 at δ 6.07 (1H, dd, J = 14.5, 11.0 Hz) and H-13 at δ 5.59 (1H, m), the presences of which had already been indicated by the DAD-UV maximum at wavelength 232 nm. Finally, a singlet at δ 9.74 (1H) was indicative of an aldehydic proton H-18. Meanwhile, measurements of some methylene and methine protons were covered by peaks of the solvent (acetonitrile, δ 2.10; water, δ 4.15) and resonances of ammonium acetate (NH_4^+,δ 3.27; CH_3COO–, δ 1.84). Total accordance of the chemical shifts data and signal multiplicities of constituent 1 was compared to the LC–^{1}H NMR data of isp I (peak 1′) in BT, which served as a reference.

Thus, on the basis of the above interpretation of LC–^{1}H NMR and LC–UV-MS–MS spectroscopic data, constituent 1 was finally identified as isp I.

We also measured the LC–^{1}H NMR resonances of peak 3′, which was recognized as isp III in BT by LC–MS location [21,23–25], to see the potentiality of this technique to distinguish the difference between the structures of isp I and isp III. To our satisfaction, some notable differences could be found in the LC–^{1}H NMR spectrum of peak 3′ compared to peak 1′ (isp I) (Fig. 3b). First, a broad singlet at δ 4.92 (1H) was attributed to a downfield shifted methine proton at position C-3. Second, an additional aliphatic methyl group recorded at δ 1.27 (3H, t) was indicative of a propionyl group conjugated with the oxygen atom in position 3. The on-line data for isp I and isp III matched nearly perfectly, with the only difference in the molecular structures being a hydroxyl group at position C-3 on peak 1′ (isp I) and a propionyl unit taking the hydrogen atom place on peak 3′ (isp III). This experiment showed that LC–NMR is a powerful method to clearly display the slight differences in the fine structures of homologues.

4. Conclusion

The study presented in this paper has the advantage of combining information from independent spectroscopic data of multi-stage hyphenated techniques. It allowed the on-line structural identification of isp I produced by WSJ-2. In the strategies presented, the LC–DAD-UV and LC–ESI-MS^n were performed simultaneously, while LC–^{1}H NMR was carried out as a valuable complementary approach to ascertain the compound identity.

For the identification of known structures or for studying derivatives of a known core skeleton that are likely to be present in a given extract, LC–^{1}H NMR information is usually sufficient as shown. Spectroscopic data obtained from LC multi-coupled approaches were confirmed by one another. The combined use of these coupled techniques allows partial or complete structural identification of the main constituent(s) of an extract such as the intricate microbial secondary metabolites to be performed on-line.

It must be noted that LC–ESI-MS^n played an extremely important role in our study. It provided, besides molecular weight, structural information about fragments as large as sugar units or as small as a methoxyl or a hydroxyl substituent, by which the skeleton of the large molecule could be established. LC–^{1}H NMR was fairly successful in the on-line structural identification of macrolide antibiotics, as presented in this paper for isp I. But for molecules with characteristic ^{1}H signals that are not well separated in the NMR scale, it is still difficult to identify molecular structures on-line using this technique. In addition, molecules that exhibit very few ^{1}H signals or have mainly quaternary carbons or hydroxyl substituents in their structures will yield little structural information in LC–^{1}H NMR. And, if the detection of all the signals of the analyte is necessary, an effective alternative is to carry out the solvent suppression in two independent solvent systems such as MeCN–D_2O and MeOH–D_2O to make up for the loss of proton resonances separately.

LC-hyphenated techniques play an increasingly important role as a strategic tool to support microbial chemistry investigation. They are very useful when large numbers of metabolites may exist in the fermentation of microorganisms. Once the novelty or utility of a given constituent is established, it is important to process subsequent targeted isolation of compounds, to obtain samples for full structural elucidation and biological or pharmacological testing.

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