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Full Length Research Paper

Identification of a new strain of *Actinomadura* isolated from Saharan soil and partial characterization of its antifungal compounds

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One promising strain *Actinomadura* sp. AC170, isolated from Algerian Saharan soil, with strong antifungal activity against pathogenic and toxinogenic fungi, was selected for further studies. The 16S rRNA results showed a distinct phylogenetic lineage from the other species within the *Actinomadura* genus. The production of antibiotic substances was investigated using GYEA solid medium. The butanolic extract contained four bioactive spots detected on thin layer chromatography plates. Among these antibiotics, a complex called 170A, which showed the more interesting antifungal activity, was selected and purified by reverse-phase HPLC. This complex is composed of four compounds. Ultraviolet-visible, infrared, mass and H nuclear magnetic resonance spectroscopy studies showed that these molecules contain an aromatic ring substituted by aliphatic chains. These compounds differ from the known antibiotics produced by *Actinomadura* species.

Key words: *Actinomadura*, taxonomy, antifungal compounds, Saharan soil.

INTRODUCTION

Actinomycetes are Gram positive bacteria having a high G+C (>55%) and are well known as a rich source of bioactive molecules. They are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005).

One of the strategies to increase the possibility of discovering novel chemical entities is to screen actinomycetes considered rare in the extreme environments (Gathogo et al., 2004). Among these rare actinomycetes, the genus *Actinomadura* is reported to be the most predominant (Miyadoh, 1993). This fact shows that this genus received increasing attention and is considered as one of the most important targets in screening programs for bioactive metabolites (Dairi et al., 1999; Zakharova et al., 2003). The genus *Actinomadura* belongs to the family of *Thermomonosporaceae* (Zhang et al., 2001). Currently, it contains 37 species including 2 subspecies (Euzeby, 2010).

Previous studies have reported the abundance and diversity of actinomycetes in Algerian Saharan soils (Sabaou et al., 1998). During the course of our investigation on those soils, a strain designated AC170, was shown to have chemical and morphological properties typical of members of the genus *Actinomadura*. It is selected for its antifungal activity against various pathogenic and toxinogenic fungi (Badji et al., 2005). In this paper, we describe the identification of the isolate and the preliminary characterization of its antifungal products.

MATERIALS AND METHODS

Taxonomy of the isolate

Morphological and chemotaxonomic studies of the isolate have been reported by Badji et al. (2005).

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Physiological characteristics

For the physiological studies, 76 tests commonly used in numerical taxonomy of actinomycetes were applied. Production of melanoid pigments was tested on peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7) (Shirling and Gottlieb, 1966). Carbohydrates and organic acids assimilation as carbon source and utilization of testosterone were determined as described by Gordon et al. (1974) and Goodfellow et al. (1979). Decomposition of adenine, guanine, hypoxanthine, tyrosine and xanthine were determined by the methods of Gordon et al. (1974) and arbutin and aesculin decomposition, gelatin liquefaction, starch, Tween 80 hydrolysis and nitrate reductase production were determined as described by Marchal and Bourdon (1973). The strain was also examined for its ability to grow on glucose-yeast extract agar (GYEA) medium (Athalye et al., 1985) supplemented with 15 different antibiotics and inhibitory compounds including lysozyme (Gordon and Barnett, 1977), phenol, potassium tellurite, sodium azide, sodium chloride and violet crystal. Growth at different temperatures and pH were also tested in the same medium.

DNA preparation, PCR amplification, sequence and phylogenetic analysis

The isolate AC170 was grown on ISP2 solid medium (malt extract, 10 g; yeast extract, 4 g; glucose, 4 g; agar, 18 g; 1000 ml distilled water; pH 7.2) for 9 days at 28°C. The mycelium was scraped and washed twice with double-distilled water. About 200 mg of mycelia was used for DNA extraction as follows: the sample was dispersed in 800 µl of the aqueous lysis solution (100 mmol/l Tris-HCl, pH 7; 20 mmol/l EDTA; 250 mmol/l NaCl; 2% m/v SDS; 1 mg/ml lysozyme; qsp 100 ml H_2O). 5 µl of a 50 mg/ml RNAse solution was added and the suspension incubated at 37°C for 60 min. Ten microliters of a proteinase K solution (20 mg/ml) was added and the lysis solution was re-incubated at 65°C for 30 min. The lysate was extracted with an equal volume of phenol and centrifuged (7000 × *g*, 10 min). The aqueous layer was re-extracted with phenol (50 to 50% v/v), then by chloroform (50 to 50% v/v). DNA was recovered from the aqueous phase by the addition of NaCl (150 mmol/l final concentration) and 2 volumes of cool 95% v/v ethanol prior to centrifugation. The precipitated DNA was cleaned with 50 µl of 70% *v/v* ethanol, centrifuged (7000 × *g*, 10 min), resuspended in 50 µl of TE buffer (10 mmol/l Tris-HCl, pH 7.4; 1 mmol/l EDTA, pH 8) and stored at -20°C. The purity of DNA solutions was checked spectrophotometrically at 260 and 280 nm and the quantities of DNA were measured at 260 nm.

The 16S rDNA was amplified by using the PCR method with *Taq* DNA polymerase and primers 27f (5'–AGAGTTTGATCCT GGCTCAG–3') and 1492r (5'– GGTTACCTTGTTACGACTT–3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 98°C for 3 min followed by 30 cycles at 94°C for 1 min, primer annealing at 53°C for 1 min and primer extension at 72°C for 5 min. At the end of the cycling, the reaction mixture was held at 72°C for 5 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining.

The PCR product obtained was submitted to GenomExpress (Meylan, France) for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http://www.ncbi–nlm–nih.gov/. Phylogenetic analyses were conducted using software included in MEGA Version 3.0 (Kumar et al., 2004) package. The 16S rDNAsequence of the strain AC170 was aligned using the Clustal W program (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the genus *Actinomadura* retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the neighbour joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbour joining dataset.

Antimicrobial activity

The spectrum of activity of the strain AC170 was determined against several filamentous fungi, yeasts, Gram-positive and Gram-negative bacteria. The microorganisms are listed in Table 2. The experiments were done by streaking a straight line of the AC170 inoculum across the surface of glucose yeast extract malt extract agar (ISP2) medium on plate. After incubation for 7 days at 30°C, the bioassay organisms were streaked at right angles to the strain AC170 (straight line). The plates were incubated at 30°C and observed for antibiosis on the first and the second day. The extent of growth inhibition of the bioassay organisms was recorded by measuring the length of the inhibition range away from the strain AC170.

Isolation and purification of antimicrobial products

Strain AC170 has a particularity to grow only on solid media. The antimicrobial activity is better on GYEA (Glucose-extract of yeast agar) (Badji et al., 2005). The production of antibiotics was thus, carried out on this medium incubated during 21 days at 28°C. The 1 L of culture broth was extracted twice with *n*–butanol and was concentrated to dryness *in vacuo*. The crude extract was dissolved in methanol and applied to a thin-layer chromatography silica gel plate (Merck 60 F 254; Merck, Darmstadt, Germany), which was developed with *n*–butanol - ethyl acetate-water (3/1/1, v/v/v). The spots were detected by bioautography (Betina, 1973) on silica gel plates seeded with *Mucor ramannianus* or *Bacillus subtilis*. The active spots were also visualized under UV irradiation at 254 and 365 nm. The fraction that showed more antifungal activity was purified by semi-preparative high-pressure liquid chromatography on reversed-phase material, under the following conditions: Uptisphere UP 15 WOD C18 column (300 × 7.8 mm Interchim); mobile phase, gradient elution system of methanol-water; flow rate, 2 ml/min; UV detection at 220 nm at room temperature.

Partial characterization of products

The spectroscopic studies were made with the pure antifungal compounds. UV-visible absorption spectra were recorded on a Lambda 20 spectrophotometer (Perkin–Elmer, Wellesley, Massachusetts, USA) and an infrared spectrum was obtained on a FT-IR 1760 × spectrometer (Perkin-Elmer).

Mass spectrum was recorded on LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) equipped with a nanospray ion ESI source (negative ion mode). Proton magnetic resonance (1 H NMR) was recorded at 300 MHz using a Bruker AC-300 spectrometer (Bruker Instruments, Billerica, Massachusetts, USA).

RESULTS AND DISCUSSION

Taxonomy of the isolate

Physiologically, AC170 uses less carbonaceous sources

Table 1. Physiological characteristics of *Actinomadura* sp. AC170.

Test	Result	Test	Result
Hydrolysis of:		**Decarboxylation of sodium salts:**	
Adenine	–	Acetate	+
Aesculin	–	Benzoate	–
Arbutin	–	Butyrate	–
Casein	+	Citrate	–
Gelatin	+	Oxalate	–
Guanine	–	Propionate	–
Hypoxanthine	–	Pyruvate	–
Starch	+	Succinate	–
Testosterone	–	Tartrate	–
Tween 80	+		
Tyrosine	–	**Growth in presence of (g/l):**	
Xanthine	–	Crystal violet (0.01)	+
		Crystal violet (0.1)	–
Utilization of glucides and derivatives:		NaCl (30)	+
Adonitol		NaCl (50)	–
Arabinose			
Cellobiose	–	Lysozyme (0.05)	+
Dextrin	–	Phenol (0.5)	–
Dulcitol	–	Potassium tellurite (0.1)	+
Erythritol	+	Sodium azide (0.05)	+
Fructose	–	Sodium azide (0.1)	–
Galactose	–		
Glucose	–	**Growth at:**	
∝ –Methyl–D–glucoside	–	45 °C	–
Glycerol	+	pH 5	–
Inositol	–	pH 9	+
Lactose	+		
Maltose	+	**Growth in the presence of antibiotics (mg/l)**	
Mannitol	–	Ampicillin (75)	
Mannose	–	Chloramphenicol (25)	+
Melibiose	–	Cycloserin (25)	+
Melezitose	–	Erythromycin (10)	+
Raffinose	–	Gentamicin (5)	+
Rhamnose	–	Kanamycin (25)	–
Ribose	–	Novobiocin (10)	+
Sorbitol	–	Oleandomycin (75)	+
Sucrose	–	Oxytetracycline (25)	+
Trehalose	–	Penicillin (10)	–
D–Xylose	–	Rifamycin (5)	+
Nitrate reduction	+	Streptomycin (10)	+
Melanoid pigments	–	Sulfanylamide (75)	+
	+	Thiostrepton (75)	+
	–	Vancomycin (5)	+
		Ampicillin (75)	–

+, Positive test; –, negative test.

but resists to a great numbers of antibiotics tested (Table 1). The isolate was able to hydrolyze a few numbers of compounds such as casein, gelatin, starch, Tween 80, dextrin, glucose, glycerol, inositol, trehalose and sodium acetate. It was resistant to crystal violet (0.01 g/l), Lysozyme (0.05 g/l), potassium tellurite (0.1 g/l), sodium

Table 2. Antimicrobial activity of the strain AC170.

Bioassay organisms	Inhibition zone (mm)*
Alternaria sp.	18
Ascochyta lentis	43
Ascochyta pisi	42
Aspergillus sp.	20
Botrytis fabae	42
Botrytis cinerea	12
Cladosporium sp.	31
Fusarium culmorum	31
F. equiseti	26
F. oxysporum f.sp. *albedinis*	9
Mucor ramannianus	21
Phoma sp.	45
Pythium irregulare	14
Rhodotorula mucilginosa	5
Saccharomyces cerevisiae	18
Bacillus subtilis	34
Micrococcus luteus	27

*Each value represents the average of three measurements.

azide (0.05 g/l), sodium chloride (30 g/l) and to several antibiotics such as ampicillin (75 mg/l), chloramphenicol (25 mg/l), cycloserine (25 mg/l), erythromycin (10 mg/l), kanamycin (25 mg/l), novobiocin (10 mg/l), oleandomycin (75 mg/l), penicillin (10 mg/l), rifamycin (5 mg/l), streptomycin (10 mg/l), sulfanilamide (75 mg/l) and thiostrepton (75 mg/l).

Comparing the physiologic properties with the representative species of the *Actinomadura* genus, our isolated strain could not be assigned to any of the valid representatives listed in Bergey's Manual of Determinative Bacteriology, then it can be identified based on the molecular taxonomic studies. However, it is known that the species of the genus *Actinomadura* differ from to each other by their metabolic capacity (Toropova et al., 1985) and they are also known for their resistance to antibiotics (Terekhova et al., 1981).

Phylogenetic analysis using the 16S rDNA sequence confirmed that isolate AC170 belonged to the genus *Actinomadura* (Figure 1). The comparison between its sequence and those of the species of *Actinomadura* showed percentages of similarity lower than 97%. These percentages are very low and strongly suggest that strain AC170 is a new species. According to Stackebrandt and Gobel (1994) and Yassin et al. (2010), the species is sour to be new if the percentage of homology of the sequences 16s is lower than 97%.

Antimicrobial activity

The ability of isolate AC170 to inhibit growth of some fungi and bacteria showed very strong (> 40 mm inhibition zone), strong (30 to 40 mm), moderate (20 to 30 mm), weak (10 to 20 mm) and very weak (< 10 mm) antibiosis against some of the test organisms (Table 2). However, this isolate did not show any antibiosis activity against the gram-negative bacteria such as *Alcaligenes faecalis, Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. These results were observed within 7 days after streaking of the test organisms.

Purification and partial characterization of the antifungal compounds

On silica gel thin-layer chromatogram, the Butanolic extract was separated into four bioautographic spots with a different retention factor, which were all shown to be active against *B.* subtilis (Table 3). The spot designated as 170A (retention factor = 0.50), was found to be significantly active against *Mucor ramannianus* and *Saccharomyces cerevisiae*.

The complex 170A which showed interesting antifungal activity was selected and purified by reverse-phase high-pressure liquid chromatography. The active fractions were eluted with 80% methanol in water. 170A was composed by four components that were designated 170A1, 170A2, 170A3 and 170A4. These components were recovered separately and reinjected several times until complete purification was achieved.

Spectroscopic studies were carried out for the four compounds. The UV-visible spectra in methanol (Figure 2) for the four components showed no characteristic polyenic chromophore and exhibited the same maxima of absorption with 210 to 212, 252 to 254 and 358 nm.

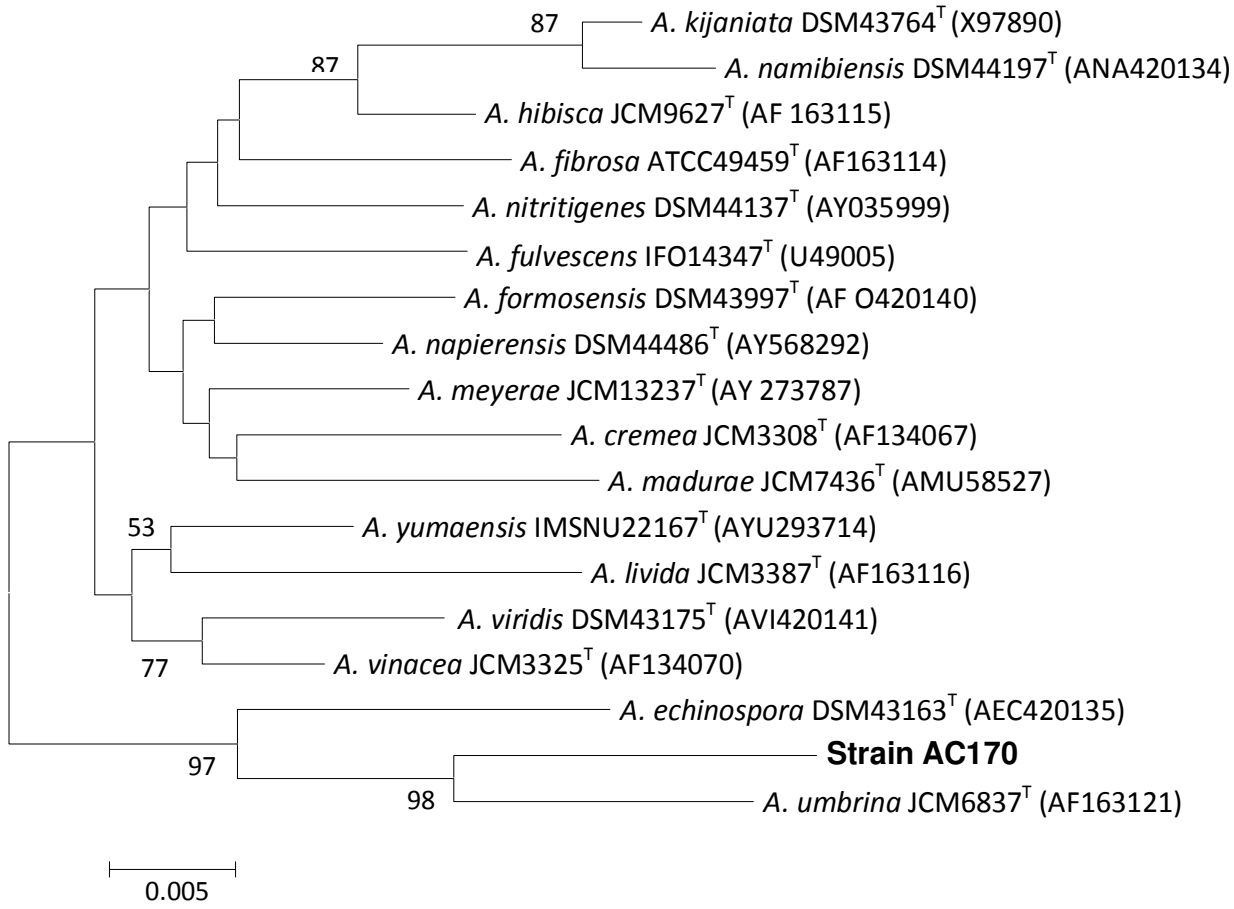

Figure 1. Phylogenetic classification of the *Actinomadura* AC170 strain based on 16s rDNA sequence. Numbers at nodes indicate percentages of bootstrap support based on a neighbour joining analysis of 1000 re-sampled datasets; only values above 50% are given. Bar: 0.005 substitutions per nucleotide position.

Table 3. Crude compounds produced by the strain AC170 on GYEA medium.

Antibiotic	Rf[a]	colour under UV (254 nm)	Activity (mm)[b]		
			M.r.	S.c.	B.s.
170^{B}	0.60	Yellow Brownish brown	25	21	33
170^{A}	0.50	Yellowish	35	30	48
170^{C}	0.38	Pale yellow	12	–	31
170^{D}	0.30	Not visible	–	–	19

M.r.= M. ramannianus (NRRL 1829); *S.c.= S. cerevisiae* (ATCC 4226); *B.s.= B. subtilis* (ATCC 6633); [a]the Rf of antimicrobial compounds were calculated after bioautography; [b]the activity was measured by a paper disc method. Values include the diameter of discs (6 mm); –, no activity.

The molecules 170A1~A4 have exactly the same spectrum IR (Figure 3). They show the following characteristics: 3411 cm^{-1} corresponds to groupings NH and/or OH. The bands from 2923 to 2853 and 1438 to 1420 cm^{-1} indicate the presence of CH, CH_2 and CH_3 aliphatic. The bands of 1621 to 1484 cm^{-1} indicate the presence of an aromatic ring. The band at 1348 cm^{-1} indicates the presence of OH function. The bands at 1199 and 1172 indicate the presence of a connection CO of the C-OH.

The mass spectrum of 170A2 is illustrated in Figure 4. It was carried out with the method of ionization chemical by desorption in positive mode and using ammonia. The "pseudo-molecular" ions products are as follows: $(M + H)^+$

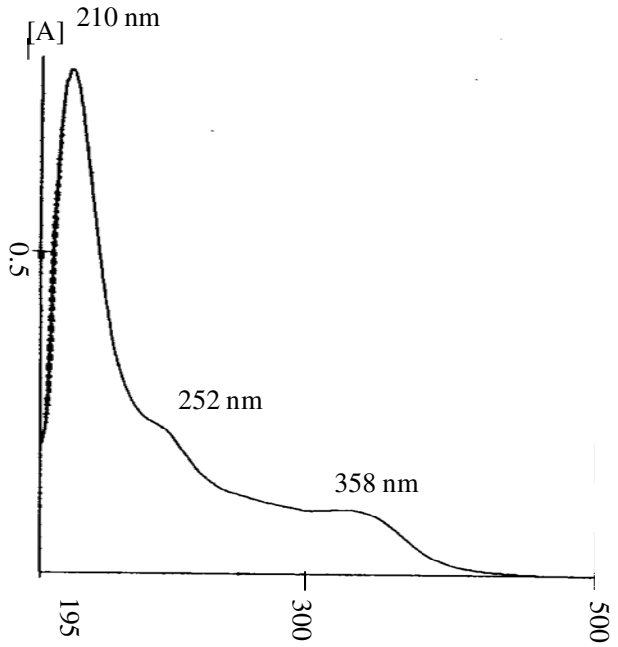

Figure 2. UV-visible spectrum of the 170A2 molecule (in methanol).

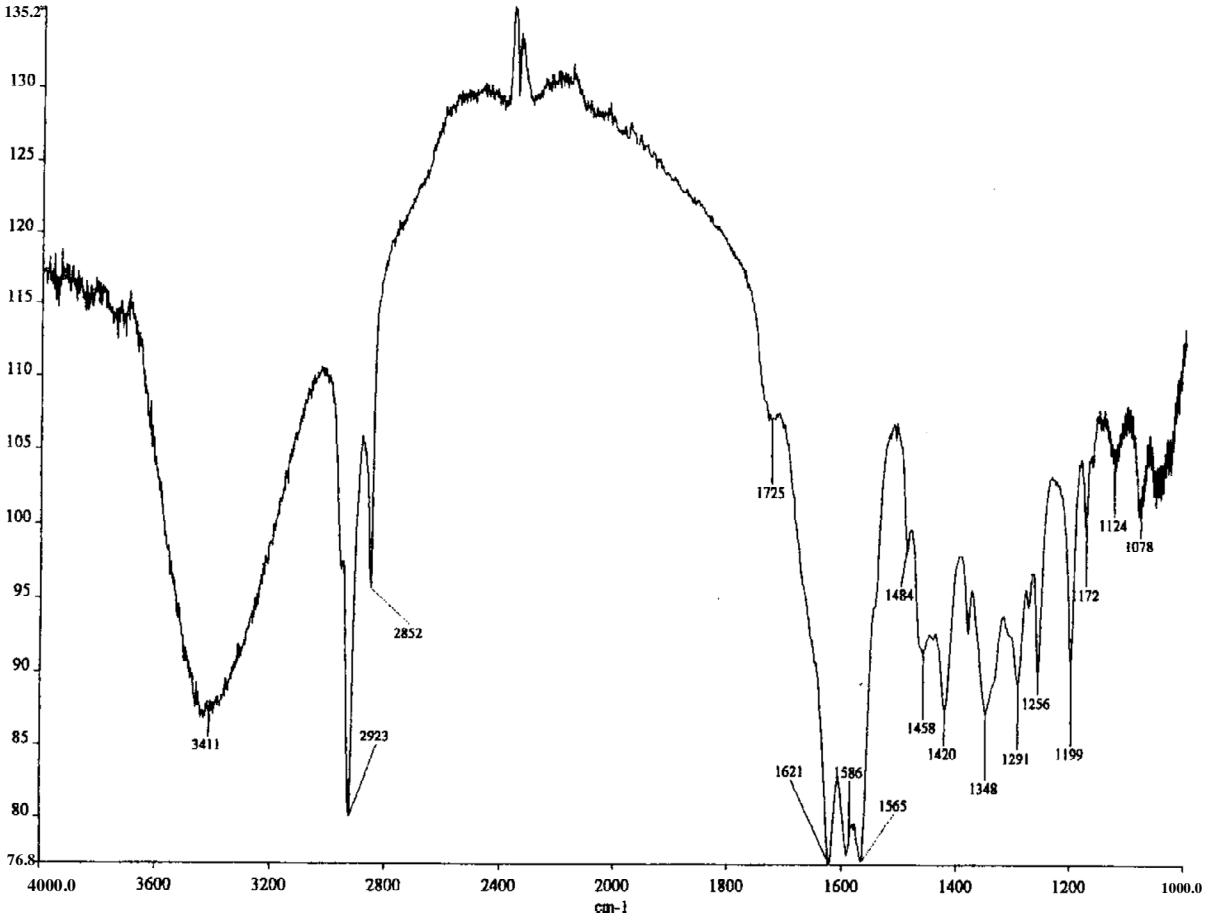

Figure 3. IR spectrum of the 170A2 molecule.

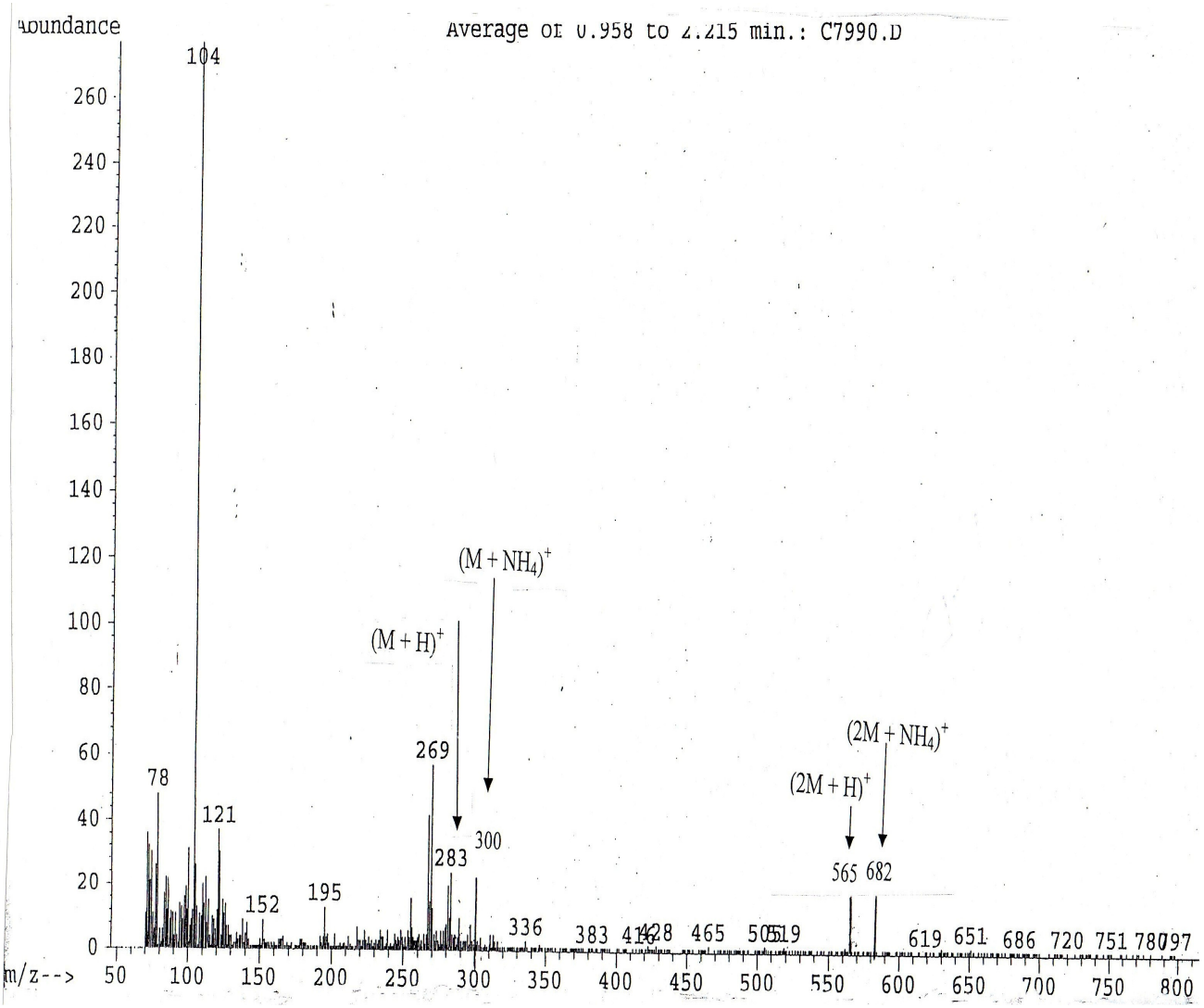

Figure 4. Mass spectrum of the 170A2 molecule (electrospray ion source).

of mass = 283, $(M + NH4)^+$ = 300, $(2M + H)^+$ = 565 and $(2M + NH4)^+$ = 582. The molecular mass of the compound is thus, 282.

H NMR showed some common main signals in the 0.7 to 3.5 ppm regions indicating the presence of alkyl groupings. The signals at 6.9 and 7.2 ppm indicate the presence of *p*–disubstituted benzenic ring (Figure 5).

Our results suggest that the antibiotics 170A1~A4 belong to the same chemical family containing a benzenic ring disubstituted by aliphatic chains. They look like those produced by a new species *Actinomadura* AC104, isolated from Saharan soil (Badji et al., 2006). This phenomenon has been reported by Terekhova et al. (1991), which observed that screening of antibiotic-producing cultures among *Actinomadura* showed that definite species mainly produced antibiotics of the same groups.

Actinomadura genus is reported to contain 37 species (Euzeby, 2010), which produce antibiotics belonging to the polyether and anthracyclin families (Berdy et al., 1987), naphtoquinone family (Kakinuma et al., 1993) and macrolactam family (Hedge et al., 1992). The antibiotics produced by the strain AC170 differ from known bioactive molecules described in the Heterocycles database (2011). However, further investigation is needed in order to determine the final chemical structure of the meta-bolites.

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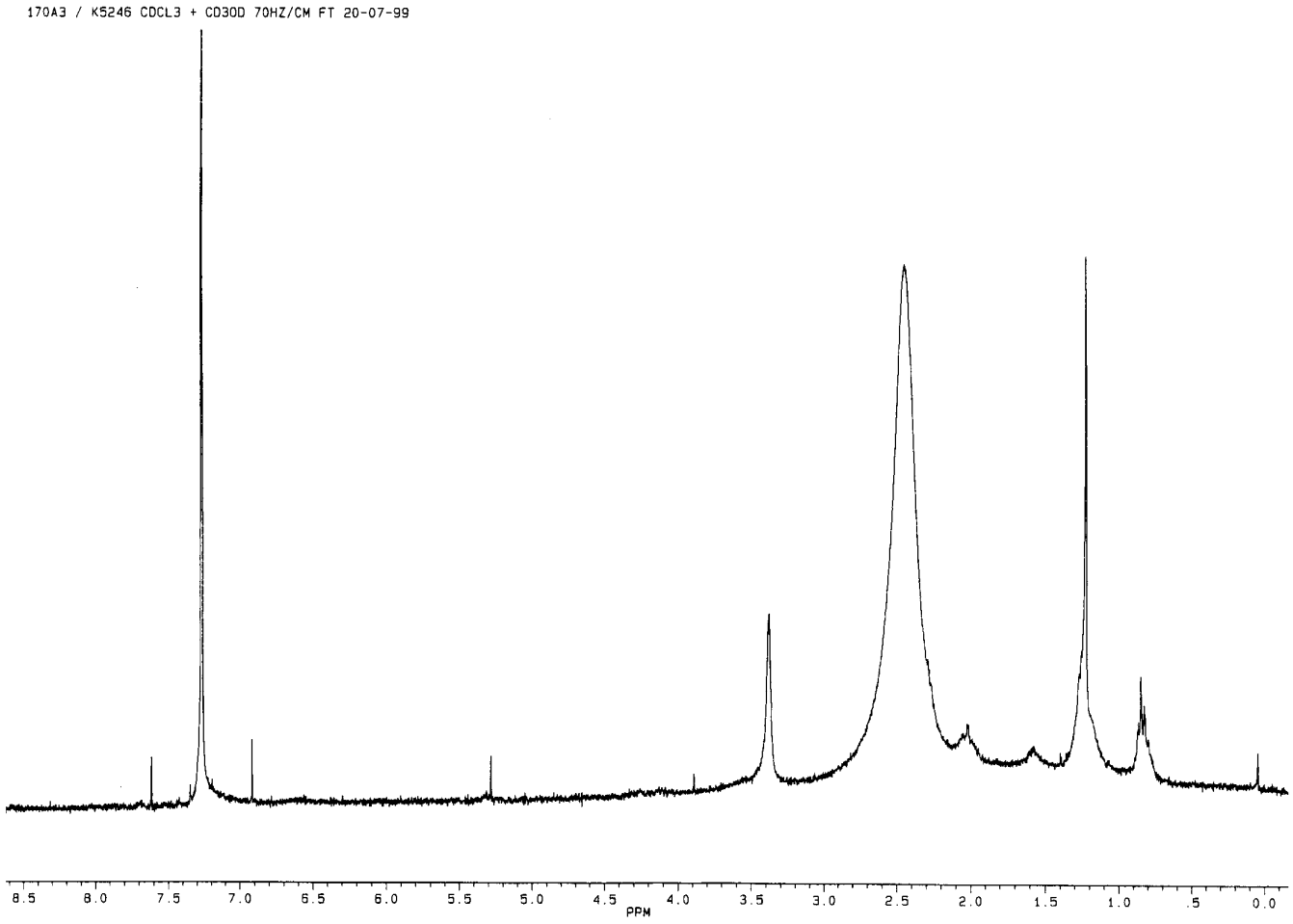

Figure 5. H NMR spectrum of the 170A2 molecule in $CDCl_3$.

564), French Embassy in Algiers."

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