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Full Length Research Paper

Isolation and characterization of *Rhodococcus ruber* CGMCC3090 that hydrolyzes aliphatic, aromatic and heterocyclic nitriles

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A bacterial strain was isolated from soil samples that had been polluted by nitrile compounds. This strain converts acrylonitrile to acrylamide with high activity. The nitrile hydrolysis activity was tested using eight substrates, including aliphatic, aromatic and heterocyclic (di)nitriles. All of the nitrile compounds were hydrolyzed by the resting cells. The main (cyano-)amide products demonstrated that nitrile hydratase was abundantly produced in this strain and that it mediated monohydrolysis. The specific conversion rate decreased in the following order: acrylonitrile > 3-cyanopyridine > valeronitrile > adiponitrile > 2,3,4,5,6-pentafluorobenzonitrile > 4-hydroxyphenylacetonitrile > 3-indoleaceto-nitrile > phthalonitrile, suggesting a higher conversion capability towards aliphatic nitriles. The strain that had broad substrate spectra was identified and named *Rhodococcus ruber* CGMCC3090 based on the 16S rDNA sequence.

Key words: *Rhodococcus ruber*, nitrile hydratase, 16S rDNA, substrate spectra, isolation, identification.

INTRODUCTION

Nitrile compounds are intermediates, products, by-products and waste products of agriculture, the chemical and pharmaceutical industries and the processing of fossil fuels (Martínková et al., 2009). The enzymatic hydrolysis of nitriles to carboxylic acids or amides plays an increasingly important role in organic synthesis and environment remediation, due to the mild reaction conditions, high activities and the high selectivities of the enzymes (Banerjee et al., 2002; Mylerová and Martínková 2003).

A wide range of prokaryotic and eukaryotic micro-organisms has been isolated for use as nitrile-hydrolyzing agents. *Rhodococcus* spp. have been reported to be the most robust, useful and versatile organisms (Bunch, 1998; Martínková et al., 2009). The most notable example of this use is the commercial production of acrylamide and nicotinamide using *Rhodococcus rhodocrous* J1 (Yamada and Kobayashi, 1996; Nagasawa et al., 1988). In addition, the immobilization of *Rhodococcus* sp. strains Novo 'SP361' and 'SP409' have been achieved comercially by NOVO Industri (DK). *R. erythropolis* AJ270, which has an amidase that has a greater rate of amide hydrolysis than of nitrile hydration, is being exploited for its enantioselectivity in organic synthesis (Wang et al., 2000; Wang et al., 2003; Ma et al., 2006; Wang JY et al., 2007; Ma et al., 2008).

Microorganisms of other different taxonomic groups have also been studied and used. *Nocardia* sp. 163, isolated from Mount Tai in the 1980's (which had the highest nitirile hydratase activity at that time), is being used to produce acrylamide at the scale of several tens of thousand tons per year at facilities in China (Zhang et al., 1998; Liu et al., 2001). Zheng and co-workers have obtained several novel, versatile nitrile-amide-converting bacteria, such as *Bacillus subtilis* ZJB-063, *Nocardia* sp.

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Abbreviations: DCW, Dry cell weight; **DMF,** N,N-dimethylformamide; **DMSO-d_6**, dimethyl sulfoxide D_6; **TMS,** tetramethylsilane; **M. P.,** melting point; **NJ,** neighbor-joining; **MP,** maximum parsimony; **TBR,** tree bisection and reconnection; **OTUs,** operational taxonomic units.

108 and *Arthrobacter nitroguajacolicus* ZJUTB06-99 (Zheng et al., 2008; Wang YJ et al., 2007; Shen et al., 2009).

However, commercial sources of nitrilases and nitrile hydratases and the appropriate nitrile-amide converting biocatalysts are almost unavailable (Chen et al., 2009). Therefore, the isolation and screening of biocatalysts that have particular characteristics in the hydrolysis of nitriles are still of great interest. In this work, we aimed at obtaining an acrylonitrile-hydrolyzing strain with broad substrate spectra. The characteristics of this strain and its capabilities in the hydrolysis of eight nitrile compounds were examined. These include: 1) the hydrolysis of aromatic nitriles; 2) the hydrolysis of heterocyclic nitriles; 3) the hydrolysis of aliphatic nitriles; and 4) the selective mono-hydrolysis of symmetric dinitriles. Furthermore, the hydrolytic profile of a perfluoronitrile, 2, 3, 4, 5, 6- pentafluorobenzonitrile, was tested using a biocatalyst for the first time. This strain, which has a broad substrate spectra was then identified based on its 16S rDNA sequence. *R. ruber* with its broad substrate spectra has demonstrated wide application potential in organic synthesis and environment remediation.

MATERIALS AND METHODS

Chemicals

Acrylonitrile and acrylamide were purchased from Sigma-Aldrich (USA). 3-cyanopyridine and 3-pyridinecarboxamide were purchased from Lonza Chemical Industry Co. Ltd (China). Phthalonitrile, valeronitrile, adiponitrile and 2,3,4,5,6-pentafluorobenzonitrile were obtained from Alfa Aesar (USA). Methanol, acetonitrile and tetrahydrofuran were of gradient grade for liquid chromatography (Merck KGaA, Germany). All other chemicals were of analysis grade. The TLC and preparative TLC were performed on silica gel GF_{254} plates that were purchased from Qingdao Marine Chemical Group Co. (China).

Sample collection and screening

Soil samples were taken from an area polluted by nitrile compounds in Tianjin, China. Nitrile-utilizing microorganisms were isolated from the soil samples by an enrichment culture method in basal medium containing 0.5 g/l glucose, 0.5 g/l KH_2PO_4 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 1.0 g/l NaCl, 13.9 mg/l $FeSO_4 \cdot 7H_2O$ and 11.9 mg/l $CoCl_2 \cdot 6H_2O$ at pH 7.2. The flasks were incubated on a rotary shaker (180r/min) at 30℃ at 3~4 d intervals, with increasing amounts of acrylonitrile (final concentrations of 0.3, 0.6 and 1.0% (v/v)) added to the sterilized medium. Then, the cultures were diluted and 100 µL aliquots were plated onto LB agar containing acrylonitrile (3.0% (v/v)) for the selection of acrylonitrile-utilizing cells. The incubation was carried out at 30℃ for 5 d, and the colonies that grew more strongly were preserved in a slant containing (g/l) beef extract 5.0, peptone 10.0, NaCl 5.0 and agar 20.0.

The cells from the slant were inoculated into 250-ml Erlenmeyer flasks containing 30 ml of medium containing 5.0 g/l glucose, 0.5 g/l KH_2PO_4 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 1.0 g/l NaCl, 13.9 mg/l $FeSO_4 \cdot 7H_2O$ and 11.9 mg/l $CoCl_2 \cdot 6H_2O$ and acrylonitrile 0.6% (v/v) at pH 7.2. The incubation was carried out as above for 48 h, at which point more acrylonitrile (3.0%, v/v) was added. Thereafter, samples were removed at 6 h intervals and acidified to pH 4.0 using 6 M HCl. The supernatant obtained by centrifugation was analyzed using GC to determine the concentrations of acrylonitrile and acrylamide. The strain with the highest conversion ratio from acrylonitrile to acrylamide was selected.

Culture of the bacteria

The cells were precultured aerobically for 28 h at 30°C in 250-ml Erlenmeyer flasks filled with 30 ml of medium, consisting of (g/l) glycerol 10.0, peptone 5.0, malt extract 3.0 and yeast extract 3.0. The cultivation was conducted at 28°C for 72 h in 250-ml Erlenmeyer flasks on a rotary shaker at 180 r/min. The medium was as follows (g/l): glucose 15.0, yeast extract 5.0, urea 7.0, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, monosodium glutamate 1.0 and $CoCl_2 \cdot 6H_2O$ 2.4×10^{-2}. The pH of both media was adjusted to 7.2 using aqueous diluted NaOH.

The resting cells were harvested from the fermentation broth by centrifugation and were washed with physiological saline buffer. Afterwards, the cells were resuspended in an appropriate volume of phosphate buffer (50 mmol/l, pH 7.5) to obtain a final concentration of 5.1 mg DCW/ml.

Hydrolysis of nitrile compounds

Due to their poor aqueous solubilities, the nitriles were first dissolved in an appropriate volume of DMF. The mixture for biotransformation consisted of 1.0 ml of resting cells, 9.0 ml of phosphate buffer (25 mmol/l, pH 7.5) and 0.5 ml (5% (v/v)) of substrate solution, giving a final concentration of 15 - 2,000 mmol/l in 100-ml Erlenmeyer flasks. The reaction was carried out at 30℃ on a rotary shaker (150r/min) for different times and terminated by addition of 6 M HCl to pH 2.5. The cells were removed by centrifugation. One part of the resulting supernatant was analyzed using HPLC. The remaining part was saturated with sodium chloride and extracted three times with ethyl acetate (at pH 8.5-9 for extraction of the amides and at pH 2-2.5 for extraction of the carboxylic acids, respectively). The combined organic layers were filtered through anhydrous Na_2SO_4. The solvent (ethyl acetate) was removed by evaporation under vacuum and the crude products were purified using preparative TLC. All of the experiments mentioned above were carried out in triplicate.

Extraction of DNA from pure cultures

Genomic DNA samples from pure cultures were extracted as described previously (Gliesche et al., 1997). The purified DNA was dissolved in 10 mM Tris-EDTA (TE) buffer, pH 8.0 (Sigma, St. Louis, Mo.). The concentration was determined spectrophotometrically and adjusted to 10µg/ml.

Amplification of the 16S rDNA

The extracted DNA (50 ng) was amplified using a pair of universal bacterial primers (Wang et al., 2002). The amplification reactions were carried out in a 50 µl reaction, containing 1×PCR Buffer (Qiagen, Inc., Valencia, CA), 20 pmol of each primer, 200 µmol/l of dNTPs, 2.5 U of *Taq* DNA polymerase (Qiagen, Inc., Valencia, CA) and sufficient ddH_2O to bring the final volume to 50 µl.

PCR was performed in a thermocycler (MJ Research Inc., USA). The PCR reaction was initiated by incubating the reaction mixture at 94℃ for 5 min, followed by 30 cycles as follows: denaturation at 94℃ for 45 s, annealing at 55℃ for 45 s and extension at 72℃ for

90 s. A final extension at 72℃ for 5 min was then followed. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis using 1×TAE buffer and subsequently reclaimed and purified using the poly-gel DNA purification kit (Sangon Co., Ltd., Shanghai, China).

Cloning and sequencing of the 16S rDNA

The PCR products were ligated into the *Bam*HI sites of the pUC_m TA cloning vector using the Qiagen PCR cloning kit (Qiagen, Inc., Valencia, CA). The recombinant DNA was transformed into *E. coli* DH 5α. The bacteria were then plated on Luria-Bertani medium containing 50 µg/ml of ampicillin, 0.5 mmol/l of IPTG (isopropyl β-D-1-thiogalactopyranoside) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indoyl-*β*-D-galacto- pyranoside) for blue/white colony screening. The plates were incubated at 37℃ for no more than 16 h and the positive, blue colonies were selected. The presence of the PCR insert was confirmed using PCR and vector-specific M13 primers. Colonies containing a PCR insert > 1,300 bp were inoculated into LB broth and grown at 37℃ with vigorous shaking (270 r/min) for approximately 16 h. Plasmid DNA was prepared using the QIAprep Spin Mini prep kit (Qiagen, Inc., Valencia, CA). The recombinants were screened by digestion with *Hin*dIII and *Kpn*I and then analyzed by electrophoresis as above. The expected PCR amplicons were sequenced (Sangon Co., Ltd., Shanghai, China).

Sequence data analysis

The sequence was assembled using Bioedit software (version 7.0.8, Tom Hall, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and submitted to GenBank. The sequence was then compared to those of previously identified organisms using a BLAST search of the GenBank, EMBL, DDBJ and PDB databases provided by the National Center for Biotechnology Information.

The 16S rRNA/DNA gene sequences used as reference sequences for phylogenetic analysis were downloaded from the databases in NCBI according to the literature of Dworkin et al. (2006) and Butler et al., (2005). The sequences were aligned using CLUSTAL X (Thompson et al., 1997). The genetic identity matrix and the distance matrix were calculated using Bioedit version 7.0.8.

Phylogenetic relations, based on the 16S rRNA/DNA sequences of more than 1,300 nucleotides (Stackebrandt et al., 1997) were reconstructed by NJ and MP analysis. The NJ analysis was performed using MEGA (Tamura et al., 2007), in which the Tamura-Nei model (Tamura and Nei, 1993) with gamma-variation was used out of consideration for the rate substitution differences between the nucleotides and the inequality of the nucleotide frequencies.

MP-based phylogenetic analyses were constructed using the software PAUP* beta version 4.0b10 (Swofford, 2002, PAUP*: Phylogenetic Analysis Using Parsimony (and other methods) 4.0 Beta, Sinauer, Sunderland) with heuristic searches using the random addition of sequences and the TBR algorithm. Support for the individual node was accessed by bootstrap resampling (Felsenstein, 1985) based on 1,000 replicates, with random additions and TBR branch swapping.

Analytical methods

The product mixtures of aromatic and heterocyclic nitriles were assayed using HPLC (Agilent 1100, USA) and measuring the absorbance at 240 nm. The mixtures were analyzed on a Kromasil 100-5 C18, 250 × 4.6 mm column using a flow rate of 1 ml/min^{-1}. The product mixtures of valeronitrile and adiponitrile were analyzed using a Shimadzu LC-20AT system (Japan) with detection by RID at 30℃. The product mixtures of acrylonitrile were analyzed using a TECHCOMP GC7890II equipped with a stainless column (Porapak Q, 2m×φ3mm) at 180℃ and detected by FID at 220℃. N_2 was used as the carrier gas at a flow rate of 40 ml/min and acetamide was used as internal standard.

ESI-MS analysis was conducted using an ion trap mass spectrometer in the negative ion mode (LCQ Advantage Max, Thermo-Finnigan, US). The sheath gas rate was 10 ARB and the ion spray voltage was 4.5 KV in full scan mode. The ^{1}H-NMR chemical shifts, referenced to TMS, were recorded on a 300 MHz spectrometer (Varian Unity Inova-300) in DMSO-d_6 at ambient temperature.

The melting point of the products was determined using an FP62 Melting Point Apparatus (Mettler Toledo).

RESULTS AND DISCUSSION

Isolation and maintenance of the strain

With the help of the enrichment method, eight isolates named ZH1-ZH8 that possessed acrylonitrile hydrolytic activity were isolated from the soil samples. Their abilities to convert acrylonitrile were tested. The ZH8 strain had the strongest ability to convert acrylonitrile to acrylamide and had a conversion ratio of 57% and a selectivity of 100% 24 h after the acrylonitrile (3%) was added. The ZH3 strain was next, with a conversion ratio of 48% and a selectivity of 90%. Accordingly, we selected the ZH8 strain for further research.

The strain ZH8 had a great strong resistance to acrylonitrile up to 20%(v/v). A moderate tolerance to acrylamide was observed, in which acrylamide reached to 400 g/l and the residual activity of nitrile hydratase still maintained 7% of the initial enzyme activity (zhang et al., unpublished). We are sure that the acrylamide-tolerance of the cells will be enhanced by delicate process optimization. The strain ZH8 showed its potential for acrylamide production.

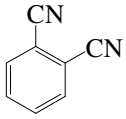
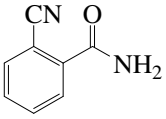
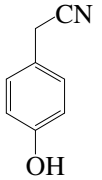
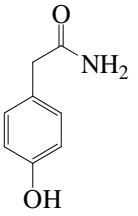
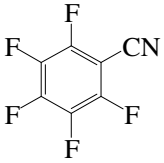
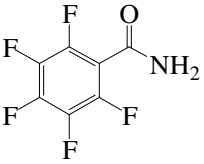
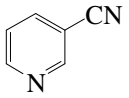
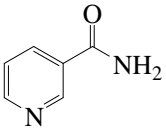
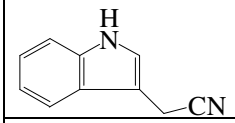
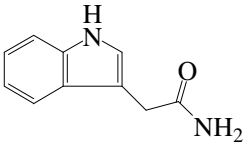
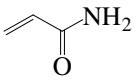
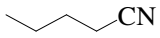
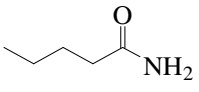
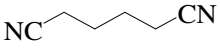
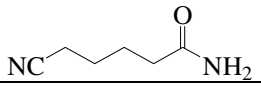
Identification of cobalt-containing nitrile hydratase

The formation of nitrile hydratase is highly enhanced by the addition of ferrous (ferric ions) or cobalt ions to the medium (Nagasawa et al., 1986; Nagasawa et al., 1988a; Nagasawa et al., 1988b). This is because nitrile hydratase has a metal active center to which the nitrile group of the substrate is bound (Sugiura et al., 1987; Brennan et al., 1996). We identified the metal active center of the nitrile hydratase as described previously (Nagasawa et al., 1988a; Wang et al., 2007). Resting ZH8 cells exhibited no detectable conversion of acrylonitrile after they were grown in cobalt-free medium for 48 h, indicating that acrylonitrile hydratase is a metalloenzyme and belongs to the cobalt NHase family.

Hydrolysis of nitrile compounds

The hydrolytic abilities of the isolate were tested using

Table 1. Hydrolytic properties of the strain ZH8.

Substrate	Initial Concentration (mmol/l)	Reaction. Time (min)	Conversionratio[a] (%)	Specific Conversion Rate[b] (mmol/(g(DCW)·min))	Main Product	Analytical Yield[c] (%)	Selectivity[d] (%)
(**1**) 	30	30	65.8	1.29	(**1a**) 	64.5	98.0
(**2**) 	30	30	85.7	1.68	(**2a**) 	83.3	97.2
(**3**) 	50	30	76.9	2.51	(**3a**) 	76.5	99.5
(**4**) 	1,000	30	70.3	45.92	(**4a**) 	70.0	99.6
(**5**) 	30	30	69.3	1.36	(**5a**) 	68.5	98.8
(**6**) 	2,000	30	75.0	98.04	(**6a**) 	74.7	99.6
(**7**) 	300	30	76.4	14.98	(**7a**) 	75.9	99.4
(**8**) 	150	30	82.3	8.07	(**8a**) 	82.3	100.0

[a]Nitrile conversion ratio was based on the initial concentration of substrate in the reaction mixture and the final concentration in the product mixture determined by HPLC or GC under the adopted condition

[b]Specific conversion rate was denoted as the amount of the substrate employed in the assay and hydrolyzed by 1 g dry cell weight (DCW) of R. ruber TCCC28001 per minute.

[c]Analytical Yield of product was based on the concentration of product in the product mixture and the initial concentration of substrate in the reaction mixture determined by HPLC or GC.

[d]Selectivity was calculated by the analytical yield of product and the the conversion ratio of the corresponding substrate.

eight nitrile compounds that are useful intermediates, including aliphatic and aromatic (heterocyclic) (di)nitriles. All the tested nitrile compounds could be hydrolyzed efficiently by resting ZH8 cells (Table 1) and the nitrile-hydrolyzing ability was substrate-dependent. The accumulation of amides or cyano-amides indicated that the bacteria abundantly produced nitrile hydratase. In addition, the cells had low amidase activity. The characterization of hydrolysis properties were grouped according to the headings that follow.

The hydrolysis of aromatic nitriles

Phthalonitrile (1) was hydrolyzed by ZH8 at a specific conversion rate of 1.29 mmol/(g(DCW)·min) (the initial concentration was 30 mmol/l). The conversion of (1) to *ortho*cyano benzamide (1a) decreased with prolonged time in all cases, indicating the formation of a small amount of some byproducts, which are more polar than (1a); although, (1a) were not identified. The presence of *Ortho*-cyano benzamide (1a) and several byproducts that were obtained in our study indicated that complicated hydrolysis had taken place, even after 30 or 60 min. The lower specific conversion rate may be attributed to steric hindrance by the bulky substituent of the cyano group adjacent to the nitrile group. The same substrate gave various products when different strains of *Rhodococcus* spp. were used. *Rhodococcus erythropolis* NCIMB (Vink et al., 2006) gave cyano-acid as the main product. The diacid was obtained using Novo SP361 (Cohen et al., 1990). *R. erythropolis* AJ270 produced *o*-cyanobenzoic acid (12% yield) and phthalimide (12% yield) and most of the phthalonitrile remained in the product mixture after 70 h (Cohn and Wang, 1997).

During the hydrolysis of 4-hydroxyphenylacetonitrile (2), the production of the main product, 4-hydroxyphenylacetamide (2a), decreased with prolonged time and a less polar byproduct ((2b), not identified) yielded up to 6.30% (60 min). (2) was converted at a specific conversion rate of 1.68 mmol/(g(DCW)·min) at 30 min when an initial concentration of 30 mmol/l was used. The low conversion rate of (2) may have resulted from the electron-donating effect and the steric distance between the CN group and the substituted aryl ring (Geresh et al., 1993).

To characterize the hydrolysis of a perfluorinated nitrile, we tested 2, 3, 4, 5, 6-pentafluorobenzonitrile (3). A specific conversion rate of 2.51mmol/ (g(DCW)·min) was obtained due to the electron-withdrawing effect of the fluorine groups in (3) (Langer et al., 2008). The 2,3,4,5,6-pentafluoro- benzamide (3a) product formed at ~100% selectivity, as the only product, even over prolonged times and elevated concentrations. This was probably because fluorobenzamide was not a substrate for the amidase (Mauger et al., 1989). Crosby et al. (1994) reported that fluorodinitriles are regioselectively converted to cyano-amide without any byproducts.

The hydrolysis of heterocyclic nitriles

3-Cyanopyridine (4) was more efficiently converted than 3-Indoleacetonitrile (5) by the resting cells. The specific conversion rates were 45.92 mmol/(g(DCW)·min) and 1.36 mmol/(g(DCW)·min), respectively. 3-pyridinecarboxamide (4a) was produced from (4) as the sole product. In addition to indole-3-acetamide (5a) as main product, (5) gave a small amount of (5b), which is presumed to be indole-3-acetic acid (Kobayashi et al., 1995; Vega-Hernández et al., 2002). It has been reported that substrate (4) was transformed to (4a) or 3-pyridinecarboxylic acid using whole cells of *R. rhodochrous* J1 when cultured with a different inducer (Nagasawa et al., 1988b; Mathew et al., 1988). However, substrate (5) was not attacked by *Nocardia* sp. NCIB 11216, a benzonitrile-utilizing organism and (4) acted as a rather poor substrate (Harper, 1977).

The hydrolysis of aliphatic nitriles

As shown in Table 1, the specific conversion rate of acrylonitrile (6) was far higher than that of valeronitrile ((7), by almost 7-fold) and adiponitrile ((8), 12-fold). The monocyano group was hydrated to form (cyano)-amide and no byproducts were observed in all cases. Further research is in progress to investigate the biotransformation of (6) for the industrial production of 5-cyano-valeramide (8a).

Pseudomonas chlororaphis B23 catalyzed (8) to form (8a) on an industrial scale and this is of important value in the synthesis of the herbicide Azafenidin (Hann et al., 1997; Shapiro et al., 2001). Another product cyano-acid was obtained from (8) by *R. erythropolis* AJ270 and *Comamonas teatosteroni* 5-MGAM-4D (Blakey et al., 1995; Gavagan et al., 1998).

The enzyme(s) involved

All the nitriles were hydrated to form (cyano-)amides as the main products, indicating that nitrile hydratase was produced abundantly. Almost no amidase or nitrilase activity was observed in the cells. *Rhodococcus* spp. have been demonstrated to possess either a bienzymatic system) of nitrile hydratase and amidase) or a trienzymatic system (Brady et al., 2004; Cohn and Wang, 1997). The enzyme system and the substrate profile vary according the inducer used in the media (Crosby et al., 1994; Wieser et al., 1998). Further investigations should be performed to determine whether nitrilase or amidase plays a role in the ZH8 strain.

Identification of the bacterium

Strain ZH8 is an aerobic, non-spore forming, non-mobile, Gram-positive bacterium. The cells form hyphae in the initial stage of the culture and then grow into rod shapes that snap into coccus elements later. The colonies were light orange-pink and turned orange after prolonged growth (Figure 1). Neither aerial hyphae nor diffusible pigments were formed.

The 16S rDNA sequence of 1,516 bp was identified and deposited in GenBank (Accession Number EU1679 12.1). A BLAST search using the megablast algorithm

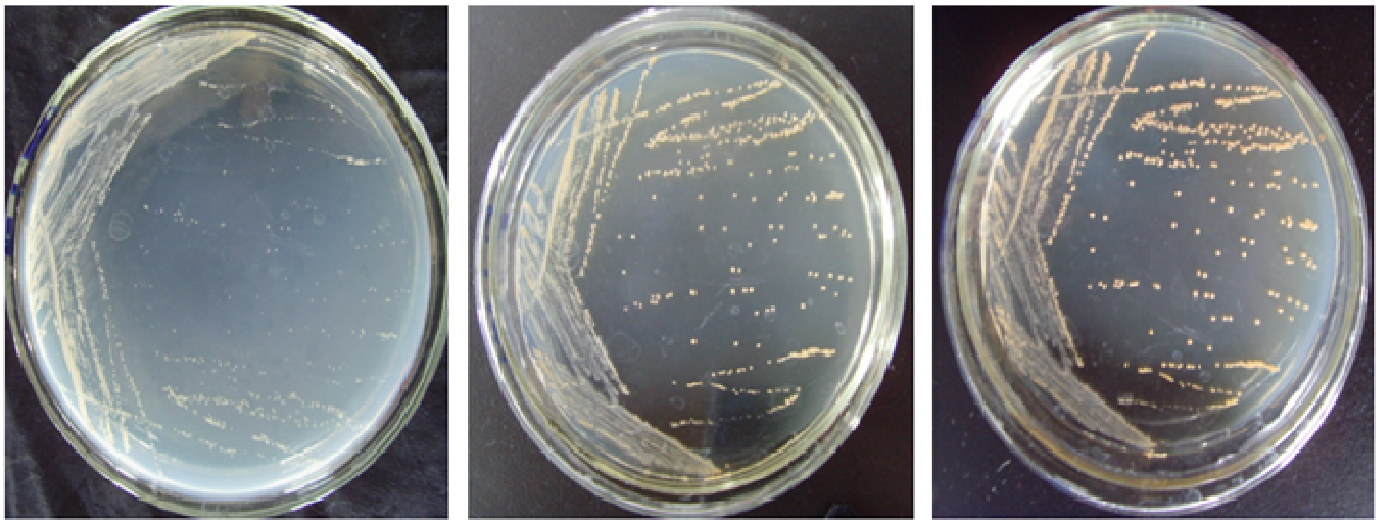


Figure 1. Colony modality of strain ZH8 cultured on LA plate (a- cultured for 48 h; b- cultured for 72 h; c- cultured for 96 h).

resulted in an identity of 100% (Query coverage 99%) between ZH8 (Max score 2800) and *Rhodococcus ruber* M2 (Accession AY247275.1, Max score 2772).

The 16S rDNA gene sequence from the ZH8 strain was compared with those from *Rhodococcus* species and representatives of other genera in the suborder *Corynebacterineae* by phylogenetic analysis using the NJ and MP methods (Figures 2 and 3). Phylogenetic analysis using the NJ method yielded a similar tree to that using the MP method, but some OTUs were positioned differently, such as *R. coprophilus*, *R. marinonascens* and the genus *Mycobacterium*. Comparisons of the nearly complete ZH8 16S rRNA sequence revealed that ZH8 was grouped with those strains of *R. ruber* with which it has 100% identity and that they together falls in the *Rhodococcus rhodochrous* 16S rRNA gene subclade (McMinn et al., 2000; Rainey et al., 1995). Nevertheless, ZH8 was less related genetically to *R. pyridinovorans* (97.3%), *R. rhodochrous* (97.0%), *Tsukamurella paurometabola* (93.8%), *Gordonia bronchialis* (93.3), *Nocardia asteroides* (93.1%), *Dietzia maris* (92.9%), *Mycobacterium immunogenum* (92.7%), *Skermania piniformis* (92.6%) or *Corynebacterium diphtheriae* (90.4%).

Accordingly, the strain ZH8 could be identified as *R. ruber* and we have deposited it in the China General Microbiological Culture Collection Center as *R. ruber* CGMCC3090. The result agreed with the conclusion that the isolation of strains of the genus *Rhodococcus* that use nitriles as the sole nitrogen or carbon sources has been found at large (Layh et al., 1997; Brady et al., 2004; Prasad et al., 2007).

The phylogeny of the genus *Rhodococcus* currently remains unresolved. In our study, the nearest genetic neighbor of *R. ruber* in the NJ tree (a clade consisting of *R. rhodochrous* and *R. pyridinovorans*) was different from that in the MP tree (a clade of *R. coprophilus*, *R. rhodochrous*, *R. pyridinovorans*, *R. phenolicus* and *R. zopfii*). Precigou et al. (2004) grouped *R. ruber* with *R. pyridinovorans* and then with *R. rhodochrous*. Furthermore, the other OTUs of *Corynebacterineae* were positioned differently by some researchers (Precigou et al., 2004; Butler et al., 2005; Gürtler et al., 2004).

Several strains of *R. ruber* have been isolated and investigated for their nitrile hydrolysis activities. Immobilized *R. ruber* cells have been demonstrated to show aliphatic nitrilase activity, which degraded acrylonitrile to ammonium acrylate (Roach et al., 2004; Hughes et al., 1998). *R. ruber* USA-AN012 (which has 99.8% identity with *R. ruber* CGMCC3090) hydrolyzed acetonitrile and acrylonitrile, in which aliphatic nitrile hydratase and amidase were involved (Brandão and Bull, 2003). *R. ruber* CGMCC3090 demonstrated nitrile hydratase activety to aliphatic and aromatic nitrile compounds and to heterocyclic nitriles.

Conclusions

A new strain, *Rhodococcus ruber* CGMCC3090, was isolated from soil samples that use acrylonitrile as their sole nitrogen source. This strain converted acrylonitrile to acrylamide at a high conversion ratio. The resting cells, when induced by urea and cobalt ions, converted a wide range of substrates that contain one or two cyano groups to the corresponding (cyano-)amides. Aliphatic (di)nitriles and 3-cyanopyridine appeared to be prior substrates. *R. ruber* with its broad substrate spectra has demonstrated wide application potential in organic synthesis and

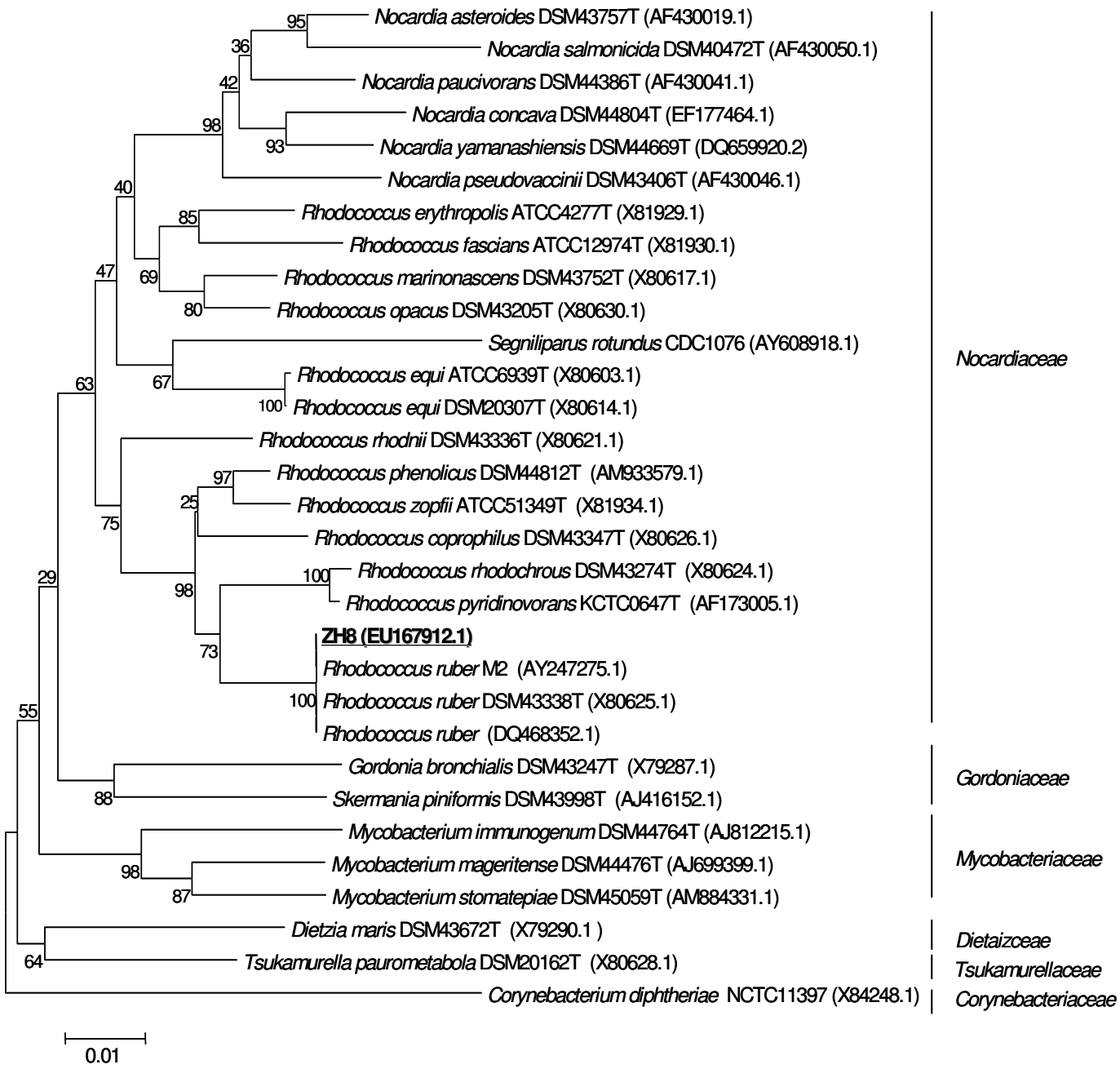


Figure 2. A phylogenetic tree based on 16S rDNA sequences constructed by the neighbor-joining method. Numerals at the nodes indicate bootstrap values (%) which were higher than 25 derived from 1000 replications. T, type strain. Bar, 0.01 substitutions per nucleotide position.

environment remediation.

ACKNOWLEDGEMENT

We sincerely thank Prof. YuGuo Zheng (Institute of Bioengineering, Zhejiang University of Technology) for his kind gift of 4-hydroxyphenylacetonitrile and 3-indoleacetonitrile.

APPENDIX

O-cyanobenzamide (*1a*, 48% isolated yield). Colorless solid. HPLC: Acetonitrile/water/ glacial acetic acid (50:50: 0.5, v/v, 2.0 ml/min), RT = 1.6 min, at 250 nm. TLC: R_f = 0.30 (chloroform/ methanol = 9:1, by vol). M.P. 168.4-169.7℃ (Keoshkerian et al., 1996). ESI-MS *m/z* 145.40 $[M-H]^-$ (Figure 4). ^{1}H-HMR (300 Hz, in DMSO-d_6) δ_H 8.18 (1 H, s, NH), 7.92 (1 H, d, *J* = 7.2Hz, H-3), 7.76 (2 H, m,

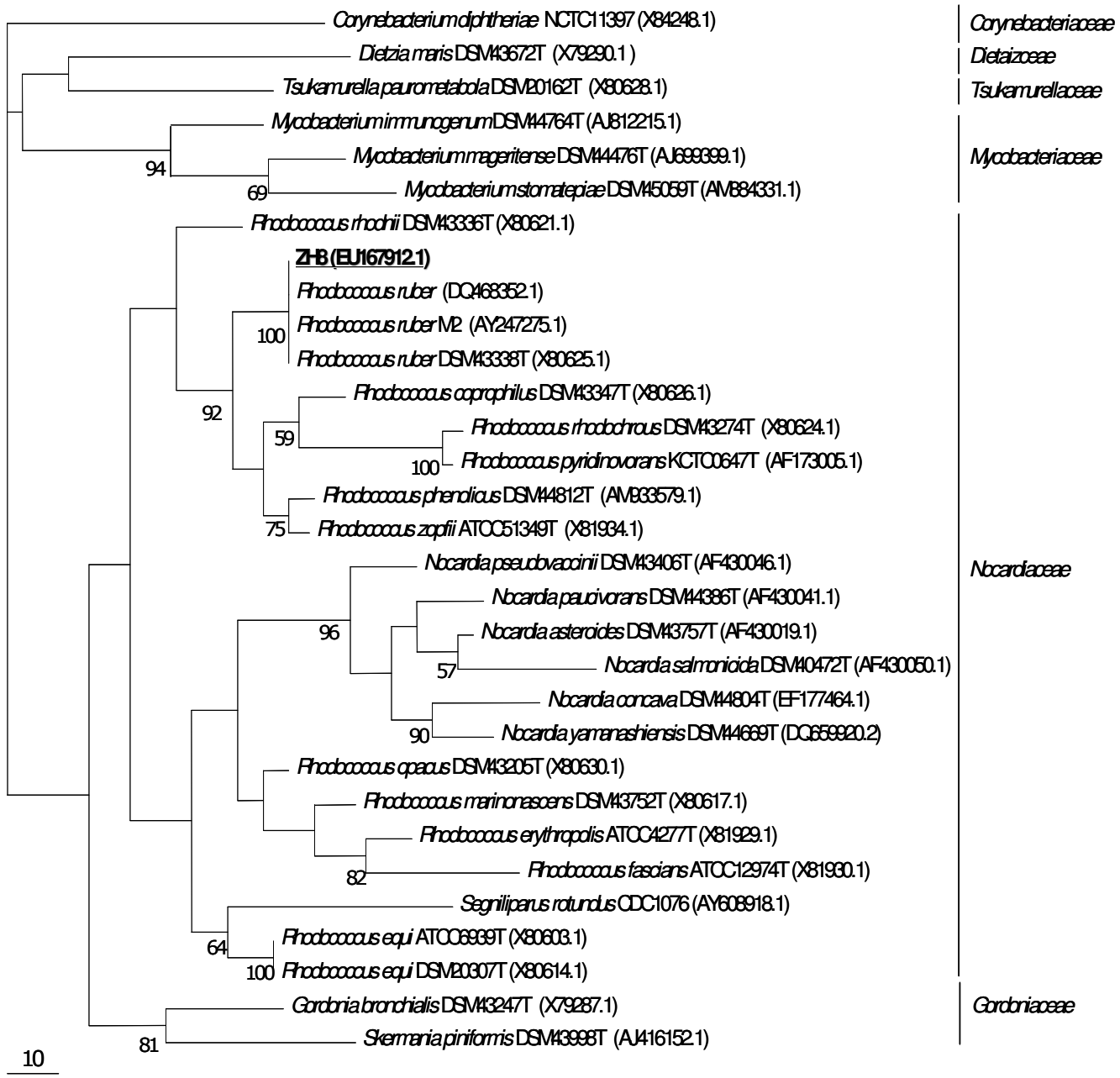
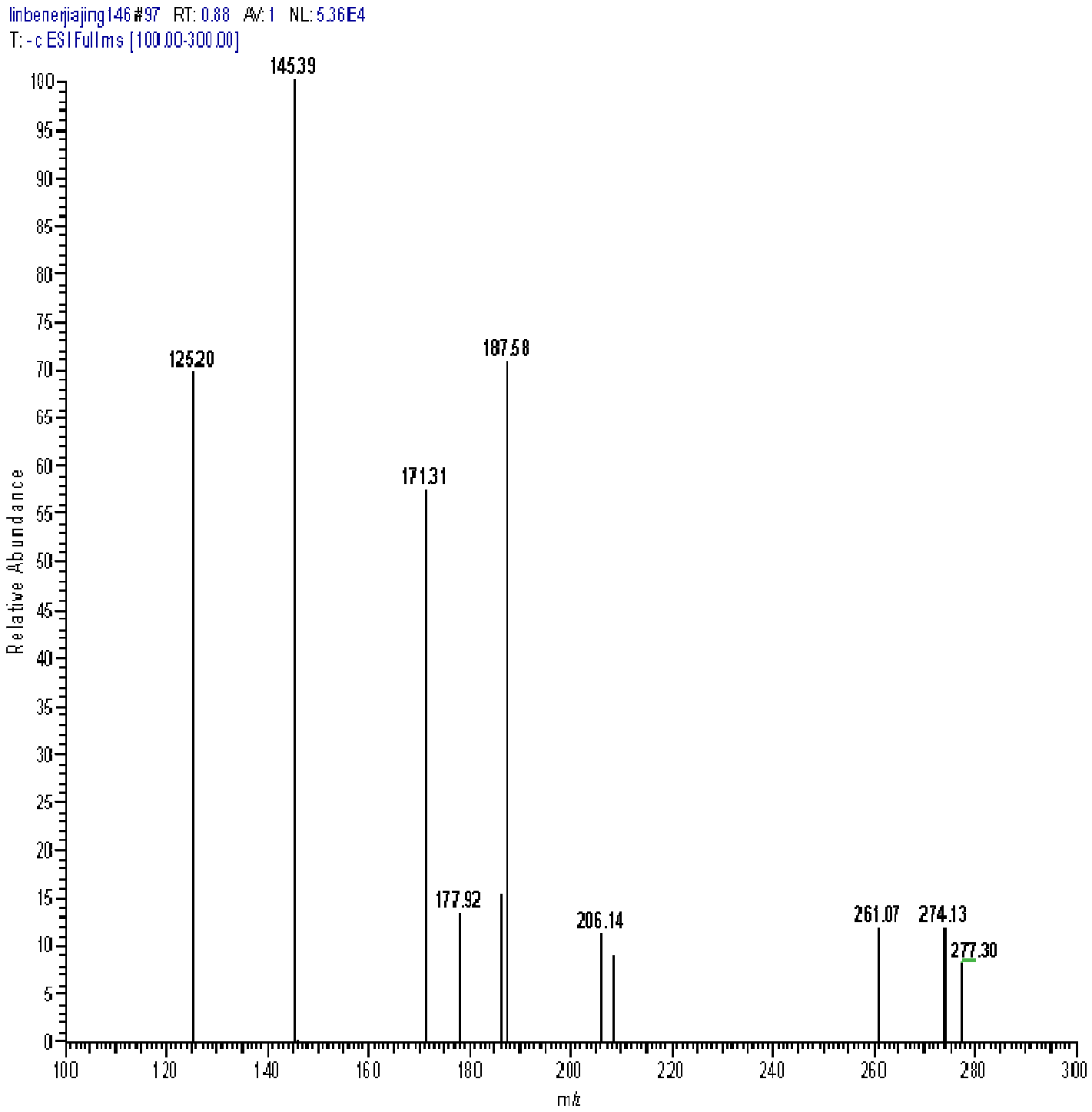


Figure 3. A phylogenetic tree based on 16S rDNA sequences constructed by the maximum parsimony method. Numerals at the nodes indicate bootstrap values (%) which were higher than 50 derived from 1000 replications. In this tree, Tree length = 901, Consistency index (CI) = 0.4506, Homoplasy index (HI) = 0.5494, Retention index (RI) = 0.5851. RC = 0.263643.

H-4, H-5), 7.74 (1 H, s, NH), 7.68 (1 H, t, *J* = 7.2Hz, H-6). *4-hydroxyphenylacetamide* (*2a*, 57% isolated yield). Colorless solid. HPLC: methanol/water /tetrahydrofuran/ glacial acetic acid (35:65:1:1, v/v, 1.0 ml/min), RT = 3.60 min, at 274 nm (Wang et al., 2007). TLC: R_f = 0.35 (chloroform/methanol = 6:1, v/v). M.P. 175.4-176.6℃ (Toshio et al., 1983). ESI-MS, *m/z* 150.40 $[M-H]^-$. ^{1}H-HMR (300 Hz, in DMSO-d_6) δ_H 9.22 (1 H, s, OH), 7.34 (1 H, s, NH), 7.03 (2 H, d, *J* = 8.7 Hz, H-2, H-6), 6.79 (1 H, s, NH), 6.67 (2 H, d, *J* = 14.1 Hz, H-3, H-5), 3.22 (2 H, s, CH_2).

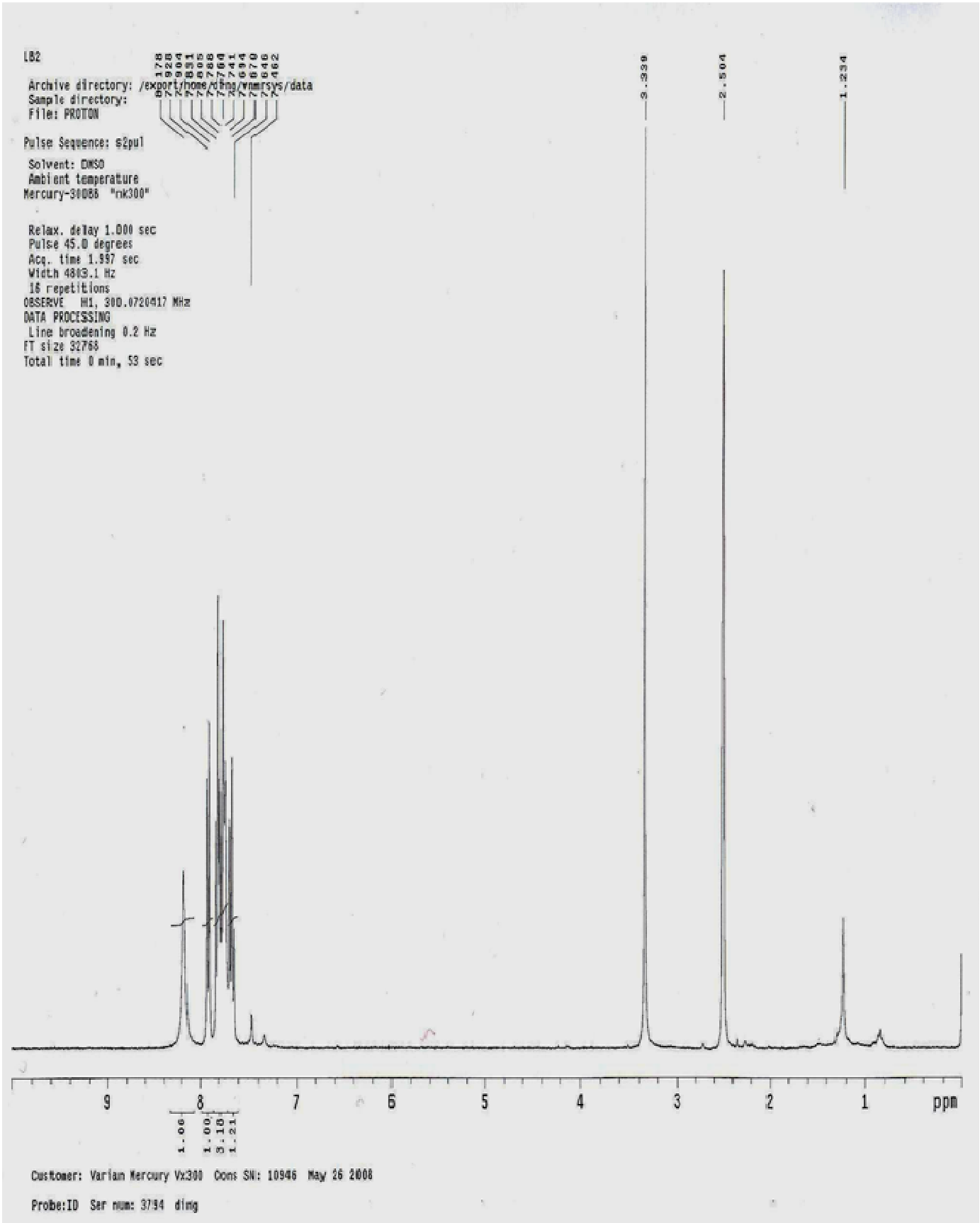
2, 3, 4, 5, 6-pentafluorobenzamide (*3a*, 52% isolated yield). Colorless solid. HPLC: acetonitrile/water /tetrahydrofuran/glacial acetic acid (80:20:1:0.5, v/v, 1.5 ml/min), RT = 1.90 min, at 250 nm. TLC: R_f = 0.30 (petroleum ether/ethyl acetate = 2:1, v/v). M.P. 147.5-148.4℃ (Utkina et al., 2002). ^{1}H-HMR (300Hz, in DMSO-d_6) δ_H 8.33 (1 H, s, NH), 8.19 (1 H, s, NH).

3-indoleacetamide (*5a*, 55% isolated yield). Pale yellow solid. HPLC: mobile phase as that of *o*-cyanobenzamide,



RT = 1.70 min, at 280 nm (Vega-Hernandez et al., 2002). TLC: R_f = 0.32 (petroleum ether/ethyl acetate/methanol = 3:3:0.7, v/v). M.P. 148.2-149.3℃. ESI-MS, *m/z* 175.40 $[M-H]^-$. ^{1}H-HMR (300 Hz, in DMSO-d_6) δ_H 10.86 (1 H, s, H-1), 7.54 (1 H, d, *J* = 7.8 Hz, H-7), 7.34 (1 H, d, *J* = 8.1 Hz, H-4), 7.29 (1 H, s, NH), 7.18 (1 H, d, *J* = 2.1 Hz, H-2), 7.06 (1 H, t, *J* = 8.1 Hz, H-5), 6.97 (1 H, t, *J* = 7.8 Hz, H-6), 6.83 (1 H, s, NH), 3.46 (2 H, s, CH_2).

Valeramide (*7a*, 46% isolated yield). White solid. HPLC: acetonitrile/water/glacial acetic acid (50:50:0.5, v/v, 1.0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16 repetitions
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7
6
5
4
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1.00
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Customer: Varian Mercury Vx300 Cons SN: 10946 May 26 2008
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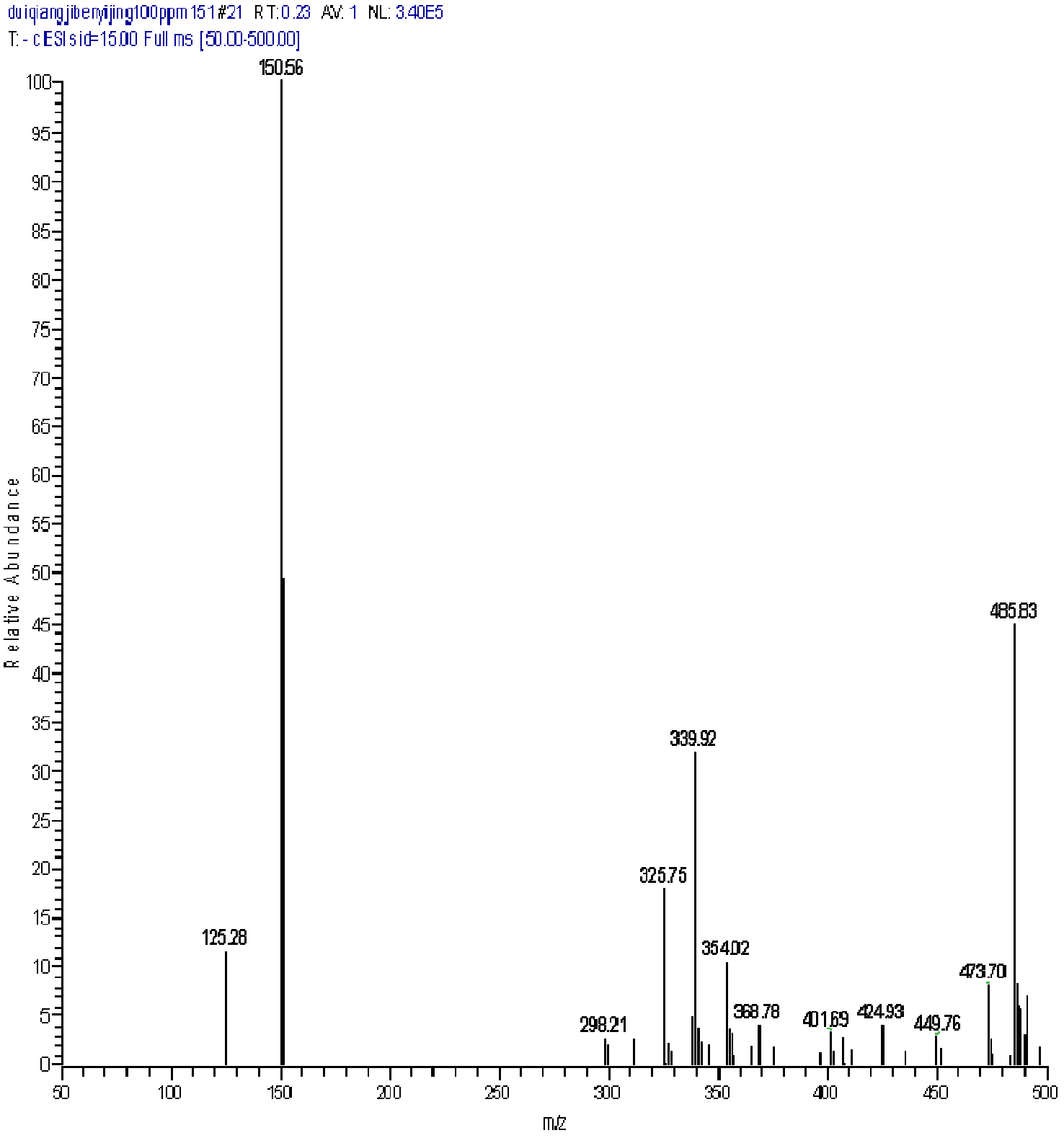
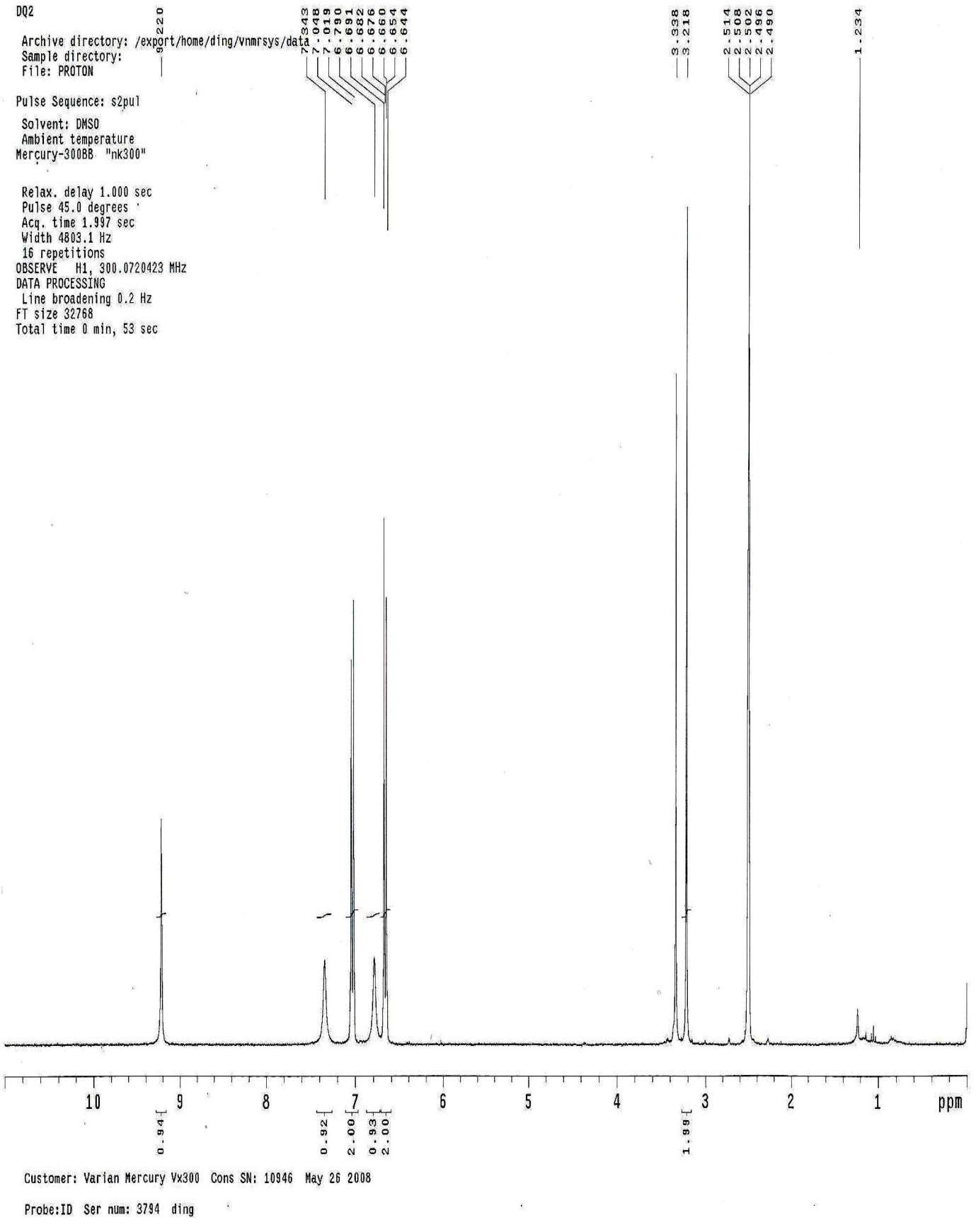


ml/min), RT = 3.60 min, detected by RID. TLC: R_f = 0.32 (petroleum ether/ethyl acetate/methanol = 2:2:0.25, v/v). M.P. 104.5-105.2℃. ^{1}H-HMR (300 Hz, in DMSO-d_6) δ_H 7.22 (1 H, s, NH), 6.68 (1 H, s, NH), 2.02 (2 H, t, *J* = 7.5 Hz, H-2), 1.45 (2 H, m, H-3), 1.26 (2 H, m, H-4), 0.86 (3 H, t, *J* = 7.5 Hz, H-5).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2.508
2.502
2.496
2.490
1.234
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9
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7
6
5
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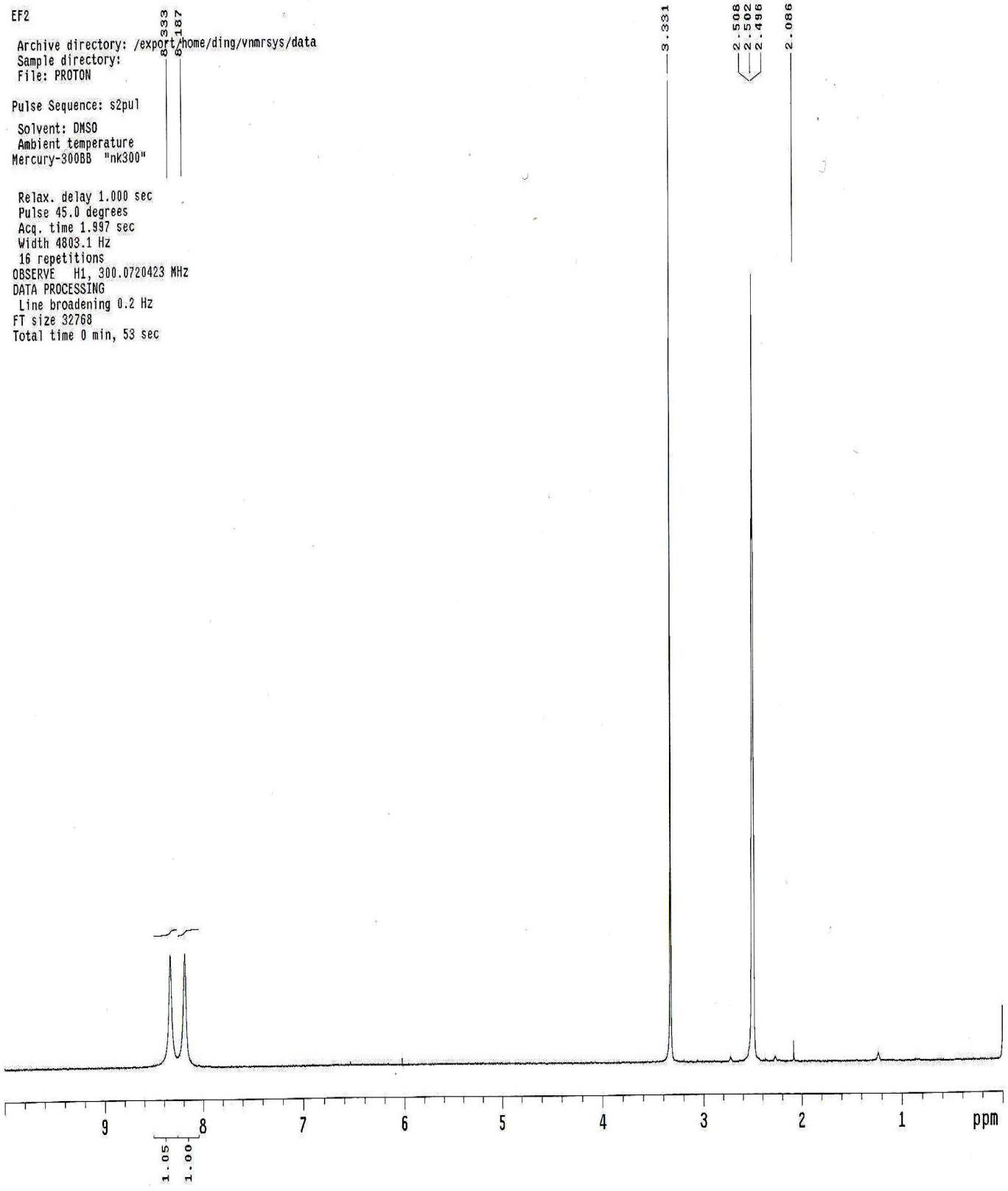
Probe:ID Ser num: 3794 ding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Pulse 45.0 degrees
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16 repetitions
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2.086
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8
7
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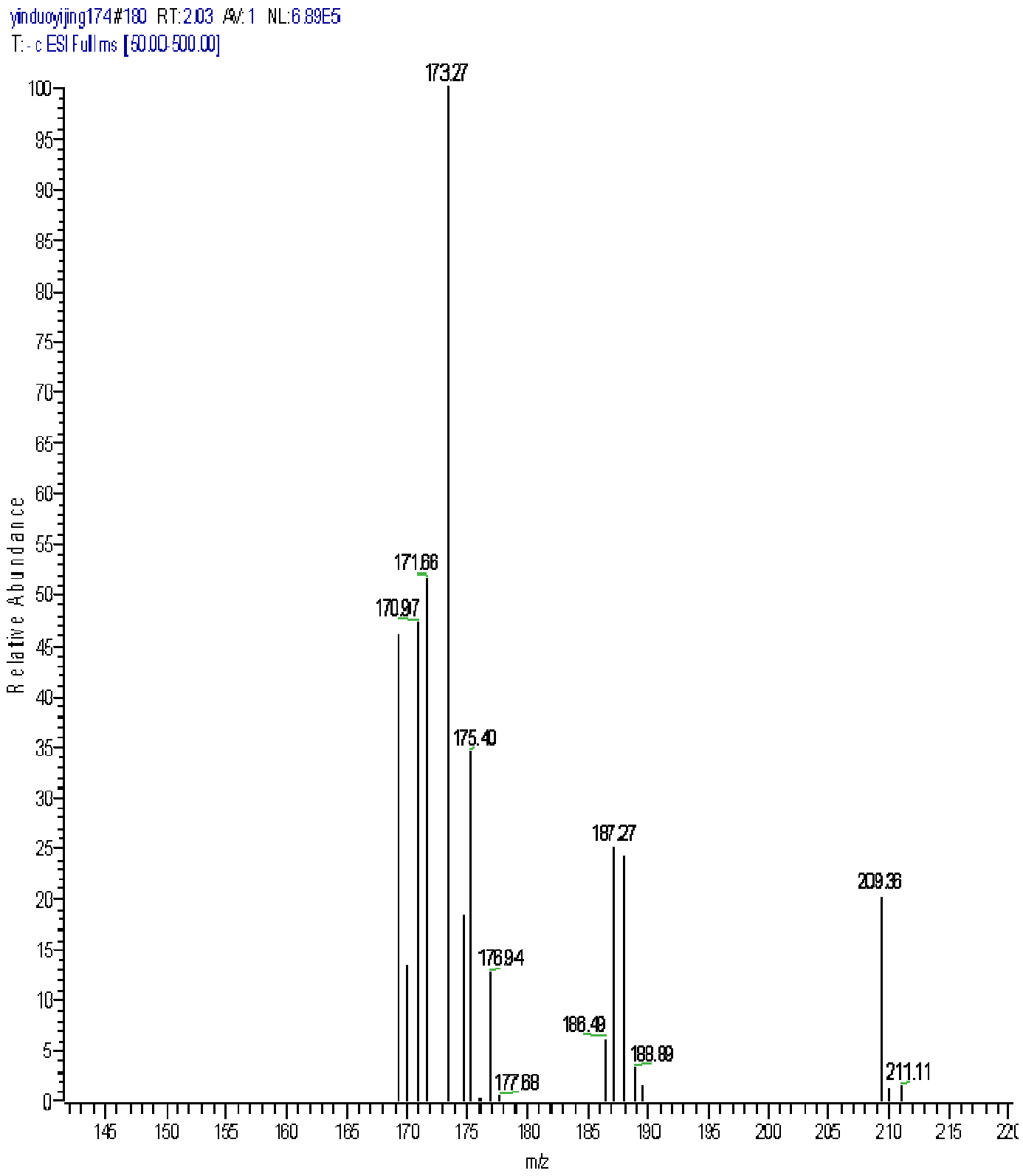
Probe:ID Ser num: 3794 ding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T: - c ESI Full ms [50.00-500.00]
173.27
171.66
170.97
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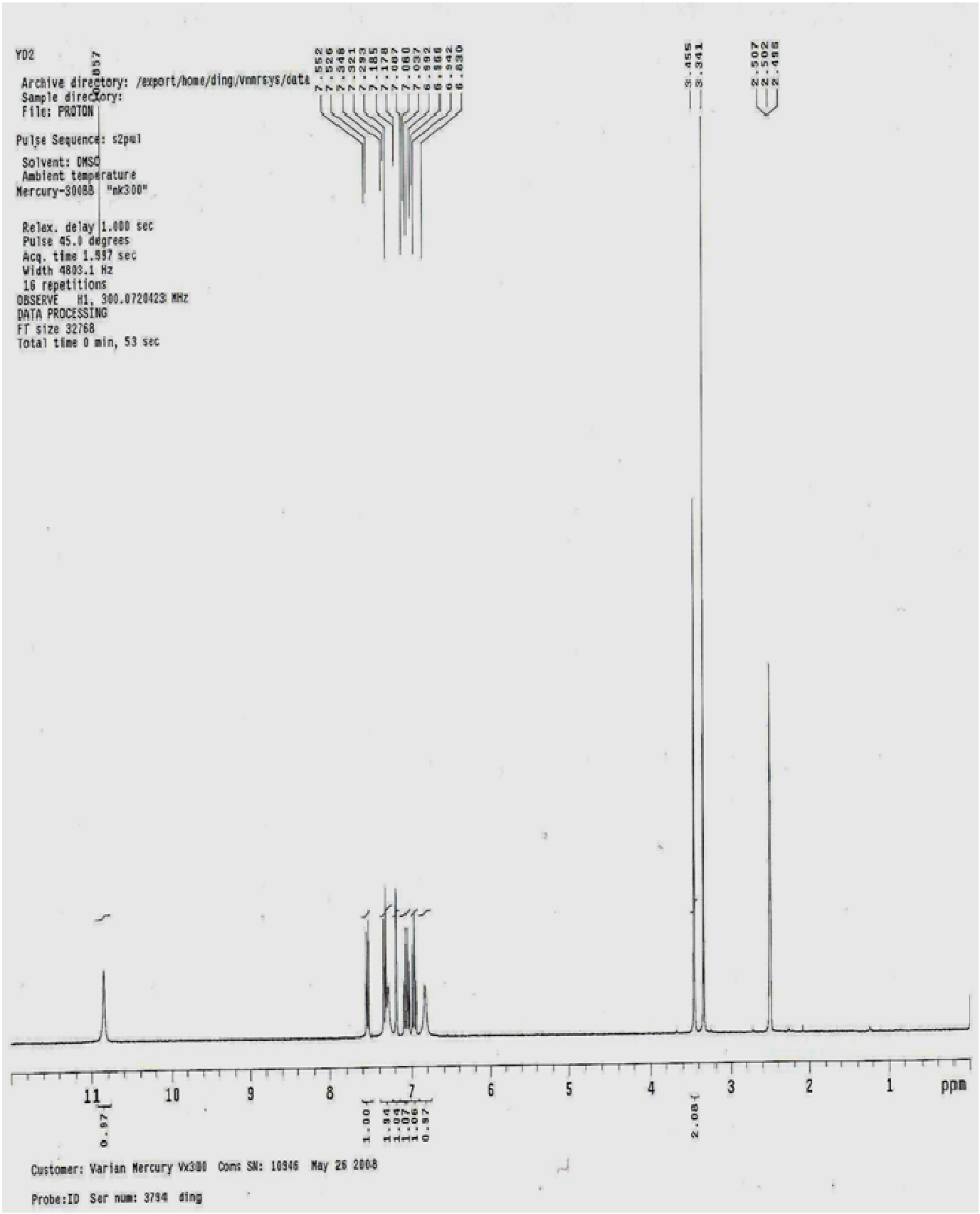
187.27
209.36
176.94
186.49
188.89
177.68
211.11
Relative Abundance
m/z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7.060
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6.956
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2.507
2.502
2.496
11
10
9
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7
6
5
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3
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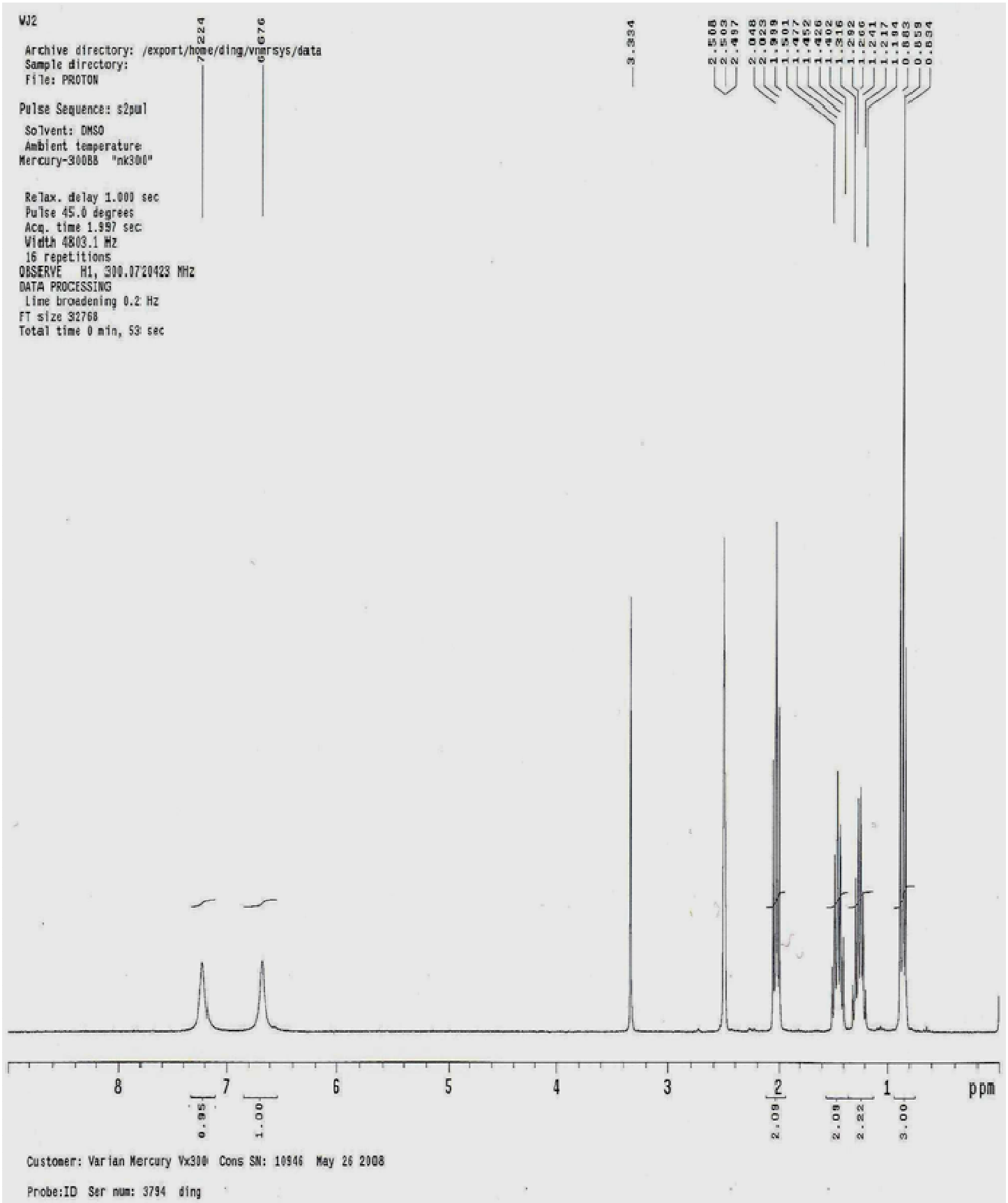
Probe:ID Ser num: 3794 ding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1.402
1.316
1.292
1.266
1.241
1.217
1.194
0.883
0.859
0.834
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7
6
5
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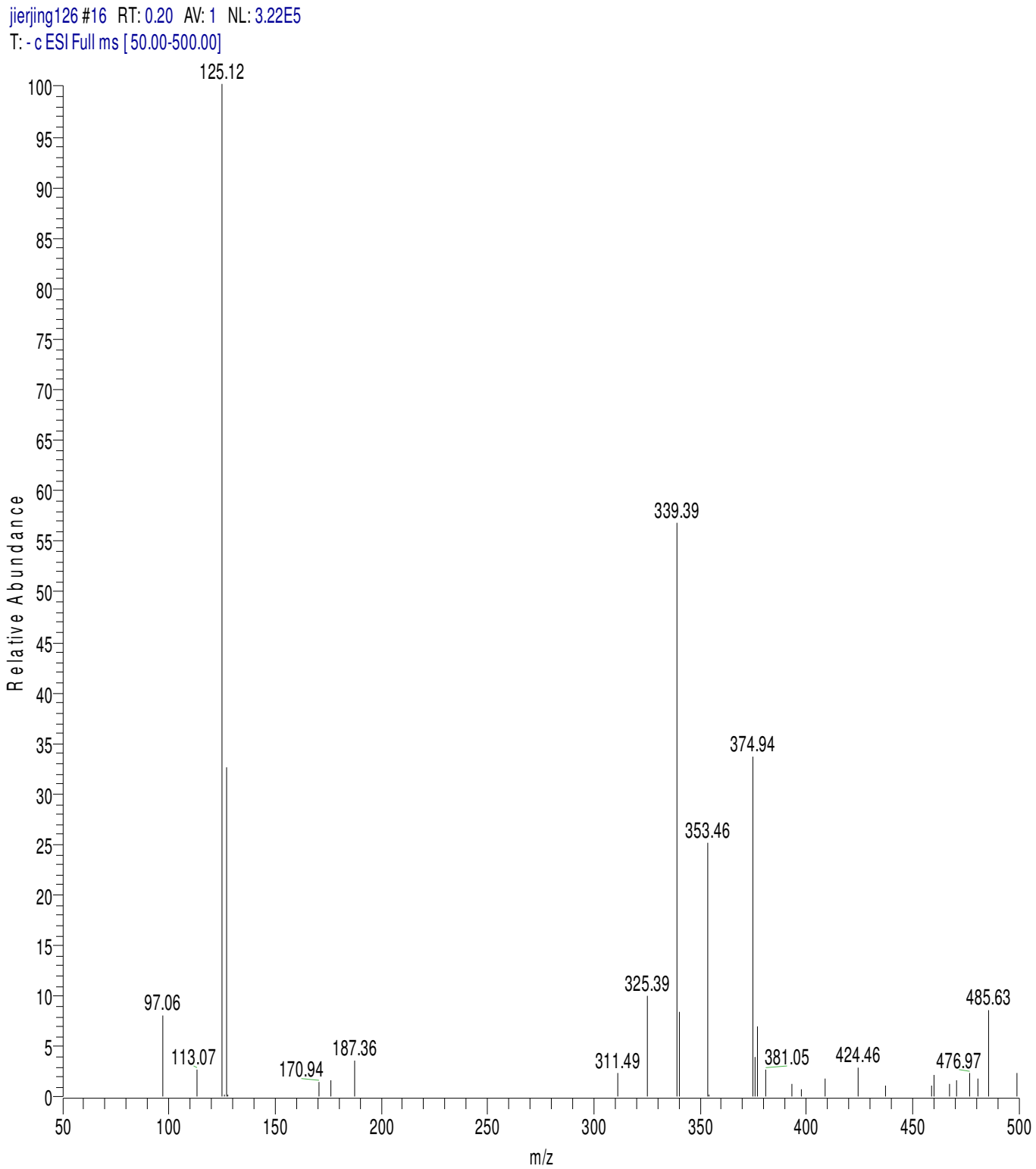
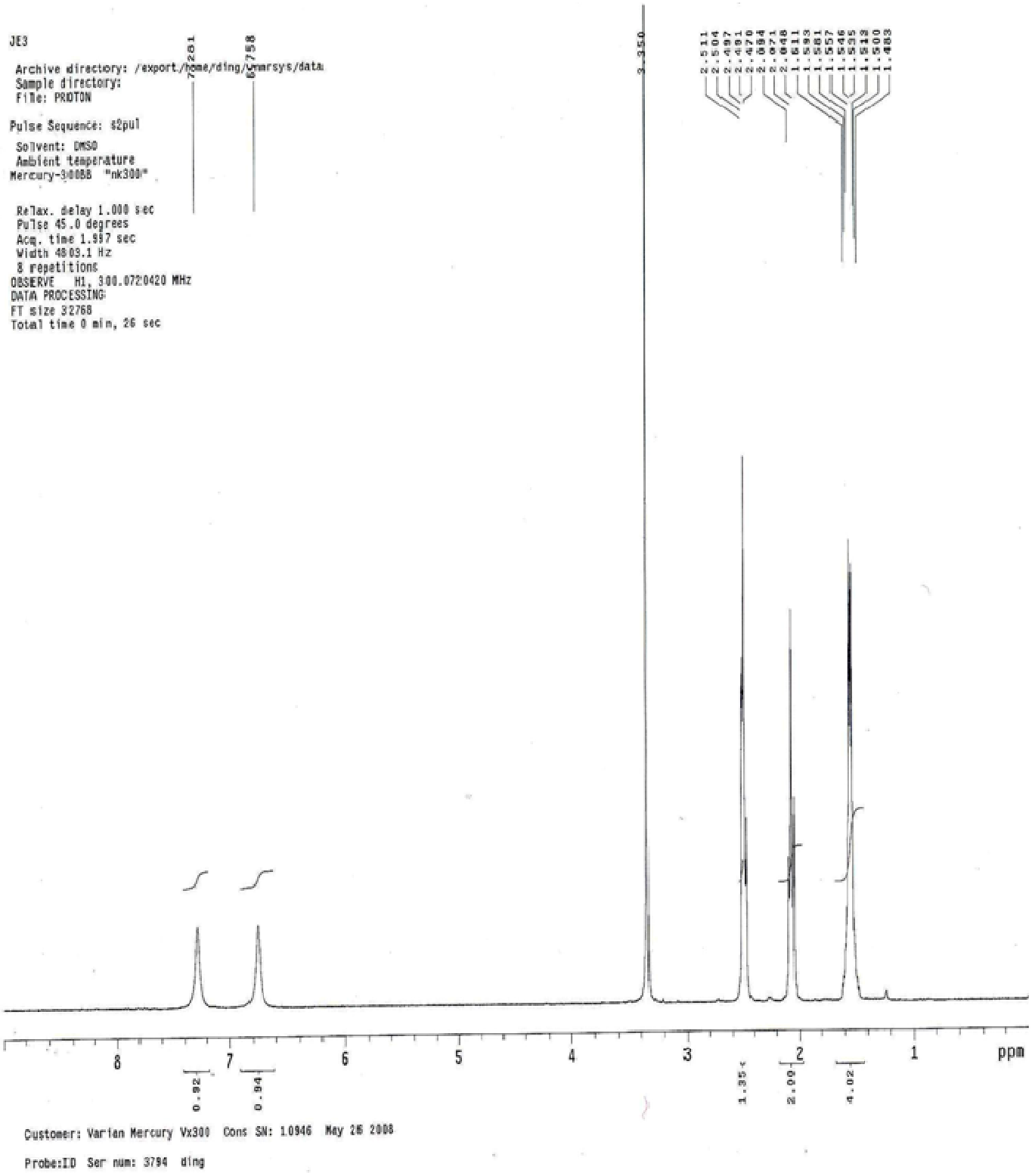


5-cyanovaleramide (*8a*, 55% isolated yield). Pale yellow solid. HPLC: same as that of valeramide, RT = 3.06 min. TLC: R_f = 0.30 (petroleum ether/ethyl acetate/ methanol = 2:2:0.8, v/v). M.P. 64.2-65.4℃ (Witzel et al., 1994). ESI-MS, *m/z* 125.17 $[M-H]^-$. ^{1}H-HMR (300 Hz, in DMSO-d_6) δ_H 7.28 (1 H, s, NH), 6.76 (1 H, s, NH), 2.49 (2 H, t, *J* = 6.3 Hz, H-5), 2.07 (2 H, t, *J* = 6.9 Hz, H-2), 1.55 (4 H, m, H-3, H-4).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