Talanta 123 (2014) 89–94

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Structural characterization of cyclosporin A, C and microbial bio-transformed cyclosporin A analog AM6 using HPLC–ESI–ion trap-mass spectrometry

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ARTICLE INFO

Article history:
Received 2 January 2014
Accepted 31 January 2014
Available online 8 February 2014

Keywords:
Cyclosporin analog
HPLC–ESI–ion trap-MS^n analysis
(γ-hydroxy-MeLeu^6)CyA
MS fragmentation patterns

ABSTRACT

Cyclosporin A (CyA), a cyclic undecapeptide produced by a number of fungi, contains 11 unusual amino acids, and has been one of the most commonly prescribed immunosuppressive drugs. To date, there are over sixty different analogs reported as congeners and analogs resulting from precursor-directed biosynthesis, human CYP-mediated metabolites, or microbial bio-transformed analogs. However, there is still a need for more structurally diverse CyA analogs in order to discover new biological potentials and/or improve the physicochemical properties of the existing cyclosporins. As a result of the complexity of the resulting mass spectrometric (MS) data caused by its unusual amino acid composition and its cyclic nature, structural characterization of these cyclic peptides based on fragmentation patterns using multiple tandem MS analyses is challenging task. Here, we describe, an efficient HPLC–ESI–ion trap MS^n (up to MS^8) was developed for the identification of CyA and CyC, a (Thr^2)CyA congener in which L-aminobutyric acid (Abu) is replaced by L-threonine (Thr). In addition, we examined the fragmentation patterns of a CyA analog obtained from the cultivation of a recombinant *Streptomyces venezuelae* strain fed with CyA, assigning this analog as (γ-hydroxy-MeLeu^6)CyA (otherwise, known as an human CYP metabolite AM6). This is the first report on both the MS^n-aided identification of CyC and the structural characterization of a CyA analog by employing HPLC–ESI–ion trap MS^n analysis.

1. Introduction

Cyclosporins are a group of closely related cyclic undecapeptides produced as secondary metabolites by a number of fungi, mainly from *Tolypocladium inflatum* [1,2]. Cyclosporin A (CyA) is the main component of this family of cyclic peptides containing 11 unusual amino acids (Fig. 1). CyA was first isolated as an antifungal antibiotic [1], but was later discovered as immunosuppressive agent [3]. Since its 1983 approval by the FDA for clinical use for the prevention of graft rejection in transplantation, CyA has been one of the most commonly prescribed immunosuppressive drugs for the treatment of patients with organ transplantation and autoimmune diseases [4].

Several natural CyA congeners with substitutions on the ring structure have been isolated and identified (CyB to CyI, CyK to CyZ and Cy26 to Cy32) so far [5]. Eighteen of the 32 analogs have a single alteration with respect to amino acid exchange or lack of *N*-methylation (i.e. CyC as (Thr^2)CyA in which L-aminobutyric acid (Abu) is replaced by L-threonine (Thr)), whereas fourteen are doubly modified [5]. (Fig. 1) The structures of these congeners have been determined by spectroscopic investigation and/or hydrolytic cleavage followed by the identification of amino acid profiles.

In addition to the above-described naturally occurring cyclosporins, some cyclosporin analogs were produced by precursor directed biosynthetic approaches. Feeding L-leucine (Leu) to the CyA-producing fungus produced a new analog (MeLeu^1)CyA, namely CyJ which differs in position 1 from CyA [6]. The addition of DL-α-allyl glycine (allylGly) to the culture media of fungus generated (allylGly^2)CyA. An exogenous supply of D-serine (Ser) led to the production of (Ser^8)CyA. CyC, CyD and CyG [7]. Also, there was a report for the biosynthesis of ring-extended cyclosporine analogs [8]. The introduction of β-alanine (β-Ala) into position 7 or 8 of the ring instead of the α-alanine makes 34-membered rings of CyA, such as (β-Ala^7)CyA and (β-Ala^8)CyA. CyA could also be metabolized in human by cytochrome P450 hydroxylase system CYP3A, to more than 30

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http://dx.doi.org/10.1016/j.talanta.2014.01.067
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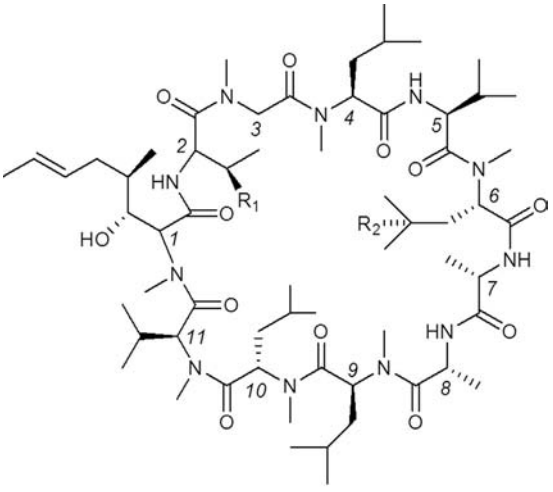

	R_1	R_2
CyA	H	H
CyC = (Thr^2)CyA	OH	H
(γ-hydroxy-$MeLeu^6$)CyA	H	OH

Fig. 1. Chemical structure of cyclosporin A (CyA), cyclosporin C (CyC) and (γ-hydroxy-$MeLeu^6$)CyA. The amino acids attached to the specific numbered positions in the backbone of CyA are listed as below; (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine[1] (=BMT^1), L-Aminobutyric acid[2] (=Abu^2), Sarcosine[3] (=Sar^3), Methyl leucine[4] (=$MeLeu^4$), L-Valine[5] (=Val^5), $MeLeu^6$, L-Alanine[7] (=Ala^7), D-Alanine[8] (=Ala^8), $MeLeu^9$, $MeLeu^{10}$, Methyl valine[11] (=$MeVal^{11}$).

metabolites [9]. The most abundant CyA metabolites are (γ-hydroxy-$MeLeu^9$)CyA, (γ-hydroxy-BMT^1)CyA, (Leu^4)CyA, and (γ-hydroxy-BMT^1-γ-hydroxy-$MeLeu^9$)CyA, which are differently annotated as AM9 (otherwise, M1), AM1 (M17), AM4N (M21) and AM19 (M8), respectively [10].

To date, several CyA analogs produced by microbial bioconversion approaches have previously been reported [11,12]; all these have been mostly isolated from large scale fermentations such as 30 L jar fermenter, and their structures were elucidated using nuclear magnetic resonance (NMR) techniques. At first, a rare actinomycete, *Sebekia benihana*, was reported to convert CyA into mono-hydroxylated analogs (γ-hydroxy-$MeLeu^4$)CyA, (γ-hydroxy-Leu^4)CyA, and di-hydroxylated one (γ-hydroxy-$MeLeu^4$-γ-hydroxy-$MeLeu^6$)CyA, with conversion yields of 35%, 4.5% and 8.6%, respectively. The structures of these analogs were thought to correspond with those of the human metabolic pathway. In 2005, the hydroxylation of CyA into AM1 and AM9 was also examined by using actinomycetes, indicating that these bioconversion patterns in actinomycete are similar to those observed for humans. Recently, the complete CYPome of the above-mentioned *Sebekia benihana* strain was constructed through *Sebekia benihana* whole genome sequencing and *in silico* analysis [13]. From these bioinformatics data, a CyA-specific P450 hydroxylase gene allowed for the conversion of CyA into (γ-hydroxy-$MeLeu^4$)CyA was identified. In particular, this hydroxylated CyA analog has been reported to have hair growth promoting potential, without any immunosuppressant activity, typical of CyA [14]. Therefore, in spite of the large volume of previous literature referring not only the biological activities of CyA and its diversified analogs, but also the structure activity relationship among them [5,15,16], there is still a need to modify the CyA structure in order to discover new biological potentials and/or improve physicochemical properties of the existing cyclosporins.

A number of high-performance liquid chromatography (HPLC) methods for quantifying CyA and/or its metabolites with ultraviolet (UV) or electrospray ionization–tandem mass spectrometry (ESI–MS/MS) detection have been reported for therapeutic drug monitoring of CyA and its metabolites in a variety of biological fluids [17–21]. HPLC coupled with ESI–MS/MS detector appears to be one of the most efficient analytical tools that are able to provide definite chemical and structural evidences diagnostic for these cyclic peptides.

Reported here is an attempt to (1) setup an efficient MS^n protocol for the structural characterization of CyA analogs using CyA and CyC, (2) generate CyA analog by supplementing CyA into a recombinant of *Streptomyces venezuelae* used as a biocatalyst, and (3) validate the fragmentation pattern of the trace amount of CyA analog, by comparison with the structurally related CyA and CyC, using ion-trap MS (up to MS^8). These detailed systematic fragmentation pathways confirm the structure of the analog as (γ-hydroxy-$MeLeu^6$)CyA (known as a human CYP metabolite AM6) (Fig. 1) [23]. Thus, this study can provide an MS/MS library for the rapid probing MS data obtained from cyclosporins and their analogs, the amount of which was insufficient for NMR analysis.

2. Materials and methods

2.1. Chemicals and materials

Methanol (MeOH), ethyl acetate (EtOAc), and water were LC grade and were supplied by J.T. Baker (Philipsburg, NJ, USA). Standards CyA and CyC, and MS-grade formic acid were obtained from Sigma (St. Louis, MO, USA). The solid-phase extraction (SPE) cartridge (SampliQ C18 ODS, 3 mL/200 mg; Agilent Technologies, Santa Clara, CA, USA) and vacuum manifold were obtained from Waters (Milford, MA, USA). All other chemicals were of the highest purity available.

2.2. Bioconversion and extraction

A recombinant strain of *S. venezuelae*, DHS2001, [24], in which the pikromycin modular polyketide synthase (PKS)-encoding gene cluster had been deleted, was grown on R2YE agar plates (40 mL/plastic Petri plate) at 30 °C for 5 days. Prior to inoculation of the recombinant strain onto the medium, CyA (200 μg dissolved in 20 μL of MeOH) was spread onto the agar surface. All bioconversion experiments were independently carried out in triplicate. The grown culture was diced and extracted with 80 mL of MeOH. The extract was pooled and concentrated under reduced pressure by means of a rotary evaporator set at 45 °C. The residues were washed with 20 mL of water and were then partitioned with an equal volume of EtOAc. The combined organic phases were concentrated again under vacuum, followed by reconstitution into 1 mL MeOH. Prior to performing the HPLC–ESI–ion trap-MS analysis, a solid-phase extraction (SPE) cleanup column was employed, not only to minimize the loss of low-level compounds of interest, but also to remove unwanted impurities such as pigments and particles, which were not readily dissolved in MeOH. The crude MeOH extracts were diluted with 9 mL of water prior to being loaded onto an SampliQ C18 ODS SPE cartridge (Agilent Technologies) which had been previously conditioned with 3 mL MeOH followed by 3 mL 10% aqueous MeOH (v/v). The column was washed with 5 mL of water and was then air-dried for about 30 s. The column was eluted with 1.5 mL MeOH, evaporated to dryness at room temperature by vacuum centrifugation, and kept in a freezer at −20 °C until analysis. For analyses, the desired residue was reconstituted to 200 μL with methanol. Twenty μL of this solution, corresponding to a culture volume of 4 mL, was subjected to HPLC–ESI–ion trap-MS^n analysis.

2.3. Method validation

The recoveries of the authentic CyA (final 1 μg mL^{-1}) spiked into a blank solid medium (R2YE agar kept at 30 °C for 5 days without microbial inoculation) were determined to check the performance of

the SPE cleanup as well as matrix effect of the media on the isolation of the CyA and its analogs during extraction and cleanup. The spiked CyA was extracted as described above and analyzed further by HPLC–ESI–ion trap MS^2. The percentage recoveries of CyA were calculated using three replicates, from a comparison of the chromatographic peak areas obtained from both spiked blank samples with those from the CyA working solutions. To evaluate the SPE cleanup procedure, the intra- and inter-day precision and accuracy were examined by analyzing CyA spiked (10, 50, and 200 ng mL^{-1}) into a blank R2YE medium with three replicates on three separate days, and are presented as the relative standard deviations (RSD).

2.4. HPLC–ESI–ion trap-MS^n

The HPLC system consisted of a Spectra SYSTEM P1000XR quaternary pump, a Spectra series AS3000 autosampler equipped with a 20 μL loop, all from ThermoFinnigan (San Jose, CA, USA). This was lined into an LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Isocratic chromatography was performed using MeOH/water (90:10) containing 0.01% formic acid at a flow rate of 200 μL min^{-1}. The MS-compatible column (XTerra MS C18, 30 × 2.1 mm^2, 2.5 μm; Waters) was operated at 30 °C over a period of 60 min. MS analysis was performed using an ESI source, and data were acquired in positive ion mode. A CyA standard solution (10 μg mL^{-1} in MeOH) was used for tuning purposes. Infusion into the mass spectrometer was performed as follows: the flow of standard solution coming from the built-in syringe pump (5 μl/min) was mixed with mobile phase (200 μL min^{-1}) through a T-piece with monitoring of the $[M+Na]^+$ sodium adduct parent ion at *m/z* 1224.9. The ESI source and MS parameters were automatically optimized and saved in a tune file, which was used during subsequent MS^n investigations. The optimized instrument settings were: capillary temperature, 300 °C; spray voltage, 6 kV, capillary voltage, 13 V; tube lens offset voltage, 120 V and with the arbitrary units for sheath gas flow and auxiliary gas flow set at 10 and 2, respectively.

Multiple tandem MS produced collision-induced dissociation (CID) spectra by trapping the $[M+Na]^+$ ion for CyA and CyC or CyA analog, for subsequent fragmentation experiments to produce characteristic product ion spectra for each cyclosporin. On the other hand, to evaluate the sensitivity and feasibility of this analytical protocol for the quantitative determination of CyA, authentic CyA spiked into a blank medium was quantified by ESI–MS^2 operated in selected reaction monitoring (SRM) mode. This was done by choosing a mass ion that was set to trace a transition of the sodium adduct ion to the product ion typical to the selected analyte: CyA, *m/z* 1224.9 > 1112.8.

3. Results and discussion

3.1. Validation

The blank media supplemented with authentic CyA at 1 μg mL^{-1} was extracted as described in "Bioconversion and Extraction" section in Experimental, and then the resulting extract was then subjected to HPLC–ESI–MS^2 analysis. The mean recovery of authentic CyA spiked into a fermentation medium was 94 ± 3%. There was a good linear correlation ($r^2 > 0.999$) between the amount of authentic CyA spiked into blank media (10, 50, 100, 150 and 200 ng mL^{-1}) and the ESI–MS^2 response (estimated as peak height) operated in SRM mode. The detection limit (LOD) was determined at 2.5 ng mL^{-1}, whereas the limit of quantification (LOQ) at a signal-to-noise ratio of 10:1 was estimated at approximately 5.3 ng mL^{-1}. Intra- and inter-day precision and accuracy using fermentation media spiked with authentic CyA at three different levels (10, 50, and 200 ng mL^{-1}) were examined. All spiked samples were extracted as described, and analyzed further by HPLC–ESI–MS^2. The precision (RSD) were all below 5%, and the intra-day and inter-day accuracy ranged from 88.2% to 94.8% and 89.2% to 92.7%, respectively (Table 1). Therefore, these data confirm that the combined use of both the organic solvent partitioning and SampliQ C18 ODS SPE cleanup together with the HPLC–ESI–MS^2 detection at nanogram level is likely to be sufficiently sensitive and reproducible for detecting CyA, its congener or analogs produced from a fermentation broth.

Table 1
Accuracy and precision of the analyses of authentic CyA spiked into a blank fermentation medium.

Concentration (ng mL^{-1})		RSD (%)	Accuracy (%)
Added	Found (mean ± SD)		
CyA (n=9)			
Intra-day			
10	8.820 ± 0.41	4.1	88.2
50	47.42 ± 2.35	4.7	94.8
200	187.7 ± 9.21	4.6	93.9
Inter-day			
10	8.920 ± 0.44	4.4	89.2
50	45.96 ± 2.16	4.3	91.9
200	185.4 ± 9.81	4.9	92.7

3.2. MS^n fragmentation studies on CyA and CyC

The fragmentation of CyA and CyC, which are commercially available, was examined for positive ion mode operation by capturing their sodium adducts $[M+Na]^+$ at *m/z* 1224.9 and 1240.9 as molecular ions, respectively. In this study, multiple tandem MS (MS^n) was used for the repeated trapping and fragmentation of ions. An advantage of the ion-trap MS instrument is the ability to switch between a full-scan MS and selected ion MS or MS^n scan of fragments, without a significant loss in sensitivity [25]. The sodium adduct ions were used as the precursor ions for multiple MS experiments. For CyA, the optimized relative collision energies (% RCE) were 49%, 57%, 51%, 48%, 40%, 29% and 22% for MS^2 to MS^8, respectively. These % RCE values were similarly applied to another standard CyC, and also to the bio-converted CyA analog. Both CyA and CyC were structurally analyzed using the following target precursor and product ion combinations in the ion-trap mass spectrometer; CyA: *m/z* 1224.9, 1112.8, 1084.8, 787.7, 518.5, 391.5, 264.2 and 142.2; CyC: *m/z* 1240.9, 1128.8, 1100.8, 787.7, 518.5, 391.5, 264.2 and 142.2.

For CyA, MS^2 gave major fragment ions at *m/z* 1206.8 $[CyA+Na-H_2O]^+$ and 1112.8 $[CyA+Na-MeVal^{11}]^+$ and MS^3 yielded a prominent product ion from the *m/z* 1112.8 fragment ion at *m/z* 1084.8, indicating the loss of methyl amino function attached onto BMT^1 (Fig. 2). MS^4 experiments, targeting *m/z* 1084.8, gave the base product ion at *m/z* 787.7, which was formed by clustered losses of residual BMT^1, together with Abu^2+Sar^3, and the subsequent loss of $MeLeu^4+Val^5$ was displayed as a main product ion at *m/z* 518.5 in MS^5 spectrum. MS^6 provided a major product ion at *m/z* 391.5, which represents the loss of $MeLeu^{10}$, and MS^7 yielded a prominent product ion at *m/z* 264.2, depicting the successive loss of the neighboring $MeLeu^9$. When targeting the ion at *m/z* 264.2, a single product ion at *m/z* 142.2 was present in the MS^8 spectrum of CyA, corresponding to the remaining $Ala^7+D\text{-}Ala^8$. Further investigation of the product ions yielded by more multiple MS^n was not possible due to the decreased intensity of the signal. These multiple tandem MS data for CyA are consistent with the reported MS^n analyses of the metal (lithium and nickel)-cationized CyA [26].

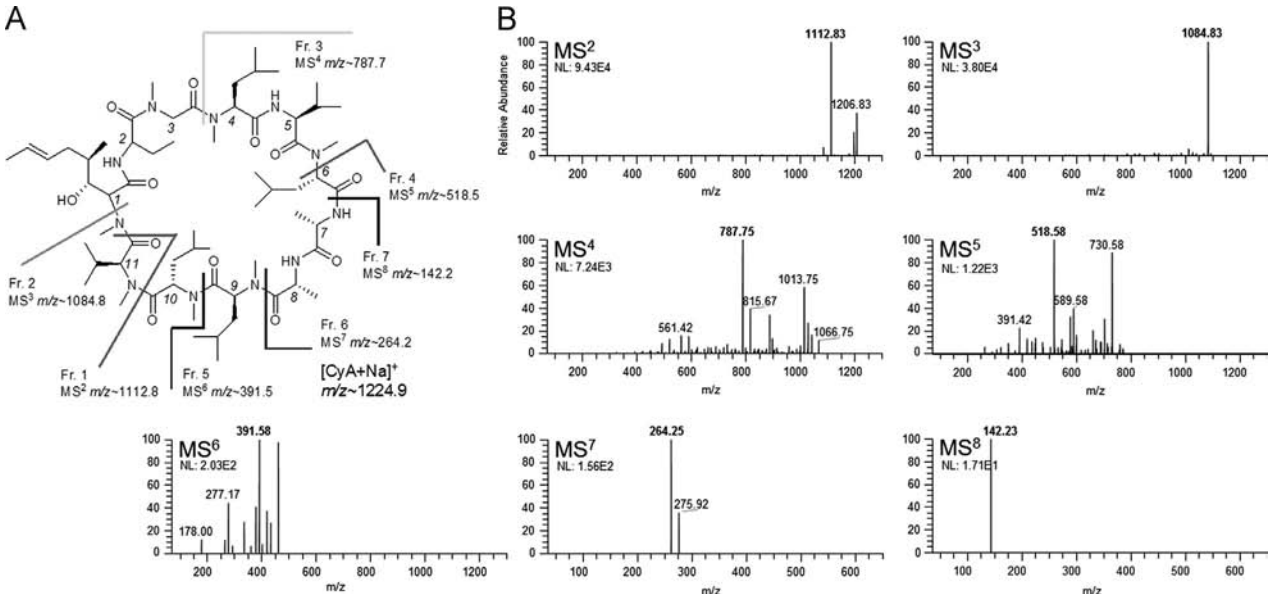

Fig. 2. Assignment of (A) fragmentation patterns of CyA and (B) its CID product ion spectra obtained from MS^2 to MS^8. Fragment ion assignments are performed for all diagnostic ions labeled below in the product ion spectrum.

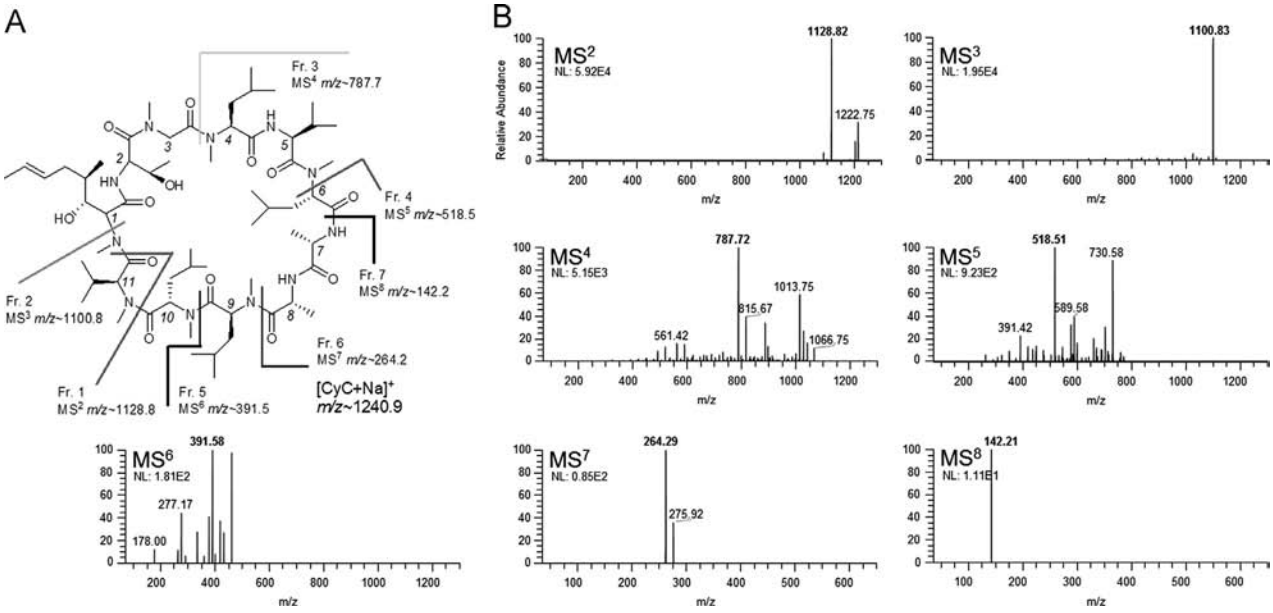

Fig. 3. Assignment of (A) fragmentation patterns of a CyA congener, CyC and (B) its CID product ion spectra obtained from MS^2 to MS^8. Fragment ion assignments are performed for all diagnostic ions labeled below in the product ion spectrum.

CyC is a (Thr^2)CyA congener in which Abu^2 is replaced by Thr^2, which means that a single hydroxyl function is appended onto the position 2 of CyA (Fig. 1). MS^2 capturing its sodium adduct at m/z 1240.9 gave a dominant fragment ion at m/z 1128.8 [CyC+Na–MeVal^{11}]$^+$, and MS^3 acquisition yielded a diagnostic product ion at m/z 1100.8, indicating the partial cleavage of BMT^1 (Fig. 3). During the multiple tandem MS analyses of MS^2 and MS^3, the values of m/z of all the annotated product ions were 16 mass units greater than that of CyA. On the other hand, MS^4 experiments afforded a typical product ion of MS^4 of CyA at m/z 787.7, which was formed by clustered losses of residual BMT^1, together with Thr^2+Sar^3. MS^5 of CyA, trapping m/z 787.7, gave a main product ion at m/z 518.5, representing the successive loss of MeLeu^4+Val^5. MS^6 and MS^7 spectra showed the main product ions at m/z 391.5 (loss of MeLeu^{10}) and m/z 264.2 (sequential loss of MeLeu^9), respectively. A single product ion at m/z 142.2 was present in the MS^8 spectrum of CyC, corresponding to the remaining Ala^7+D-Ala^8. The above-described five product ions (m/z 787.7, 518.5, 391.5, 264.2 and 142.2) generated by MS^4 to MS^8 were identical to those obtained from the same order of MS^n experiments on CyA. Therefore, based on the MS^n fragmentation patterns of both cyclosporins, structural differences between CyA and CyC are expected between the position 1 and 3 amino acids, supporting the replacement of Abu with Thr at the position 2 of CyC. This detailed structural characterization of cyclosporins could be obtained using multiple tandem MS rather than single stage MS or MS/MS. Indeed, these selected ion trapping processes could allow for an unambiguous assignment of cyclic peptides. There have been some reports on the advantages of using ion-trap MS for the characterization of cyclic peptidyl products, including CyA [22,26–28], however, this is the first

demonstration that CyC as a congener of CyA could be structurally identified using multiple MS^n experiments.

3.3. Microbial bioconversion of CyA and HPLC–ESI–MS separation

S. venezuelae DHS2001 supplemented with CyA was cultivated in R2YE solid media at 30 °C for 5 days led to a conversion of 40% of the CyA into its analog (C_1) (Fig. 4). The retention time of CyA and its analog C_1 were 42.6 and 38.0 min, respectively, by selecting the specific ions at *m/z* 1224.9 and 1240.9 as $[M+Na]^+$ from the total ion chromatogram obtained from the extract of the fermentation. As a (Thr^2)CyA congener, CyC has the same molecular ion as the sodium adduct at *m/z* 1240.9. Co-injection of CyC into the culture extract, however, indicated that CyC is eluted at 34.6 min. This implies that the bio-converted CyA analog, C_1, has an identical molecular ion peak to that of CyC, but differs in its structure. The amount of the CyA analog obtainable from the bio-conversion experiments was not sufficient to allow for further structure elucidation using NMR spectroscopic analysis. Thus, by using the MS^n protocol setup for CyA and CyC, we examined the unknown CyA analog (C_1) in the extract to validate the applicability of the MS^n platform, as well as to characterize the structure of the analog.

3.4. MS^n characterization of bio-transformed CyA analog

MS^n analysis under identical conditions to those used for CyA and CyC were performed to obtain more detailed structural information of the C_1. MS^2 targeting the sodium adduct of the analog at *m/z* 1240.9 gave a dominant fragment ion at *m/z* 1128.7 $[C_1+Na–MeVal^{11}]^+$ together with a product ion at *m/z* 1222.7 $[C_1+Na–H_2O]^+$, and MS^3 acquisition yielded a single product ion at *m/z* 1100.8, indicating the partial cleavage of BMT^1 (Fig. 5). The listed product ions observed through MS^3 analyses were identical to those from CyC, but their values were all 16 mass units more than that of CyA. MS^4 experiments of the C_1 analog provided a diagnostic product ion at *m/z* 803.5, corresponding to the clustered losses of residual BMT^1 together with Abu^2+Sar^3. MS^5 of C_1, trapping *m/z* 803.5, afforded a main product ion at *m/z* 534.5, representing the successive loss of $MeLeu^4+Val^5$. MS^6 and MS^7 spectra showed a single product ion at *m/z* 407.5 (loss of $MeLeu^{10}$) and *m/z* 280.2 (sequential loss of $MeLeu^9$), respectively. The above-described four product ions (*m/z* 803.5, 534.5, 407.5 and 280.2) generated by MS^4 to MS^7 were all 16 mass units more than those obtained from the same order of MS^n experiments on both CyA and CyC. Instead, a single product ion at *m/z* 142.2 displayed in the MS^8 spectrum of C_1 was identical to the same multiple MS acquisition of CyA and CyC. Therefore, based on the comparative profiles of fragmentation patterns found in a bio-converted CyA analog, its sequence was disclosed and modification site was located at the position 6 in CyA. To date, there could be above 60 CyA analogs reported: natural congeners, precursor directed analogs, CYP-mediated metabolites or bio-transformed analogs [5,7,11,13,23]. Among them, CyA analogs which exist as sodium adducts $[M+Na]^+$ at *m/z* 1240.9 could be listed as follows: CyC as a natural CyA congener, (Ser^8)CyA as a precursor-directed biosynthetic analog, (γ-hydroxy-$MeLeu^6$)CyA (otherwise, annotated as AM6), (γ-hydroxy-$MeLeu^9$)CyA (annotated as AM9) and (γ-hydroxy-BMT^1)CyA (AM1) as human CYP metabolites, (γ-hydroxy-$MeLeu^4$)CyA as a bio-converted analog. In particular, hydroxylation on the specific positions 1, 4 or 9 of CyA allows for the generation of a number of CYP metabolites and bio-transformed analogs. Among them, the

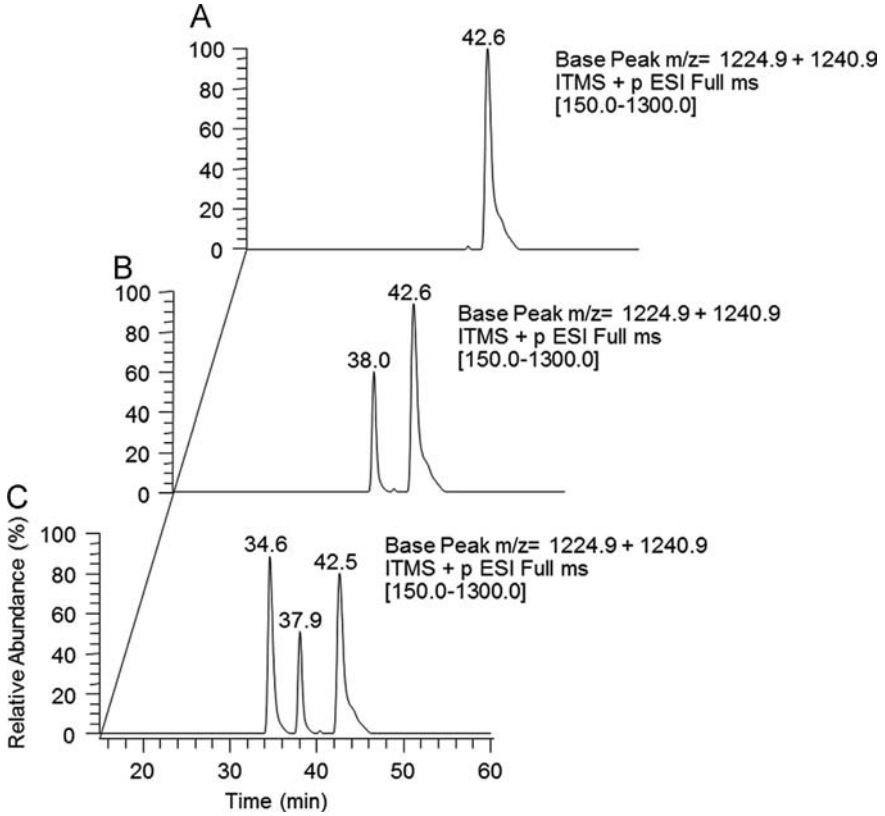

Fig. 4. Representative chromatograms of (A) the extracts of the blank media fed with CyA, (B) the extracts obtained from the fermentation of a recombinant strain of *S. venezuelae* supplemented with CyA, and (C) the above extracts spiked with authentic CyC. All were traced by the multiple selection of specific ions at *m/z* 1224.9 and 1240.9, which were identical to the sodium adducts $[M+Na]^+$ for CyA (retention time at 42.6 min), CyC (retention time at 34.6 min) and the bio-transformed molecule (retention time at 38.0 min), respectively.

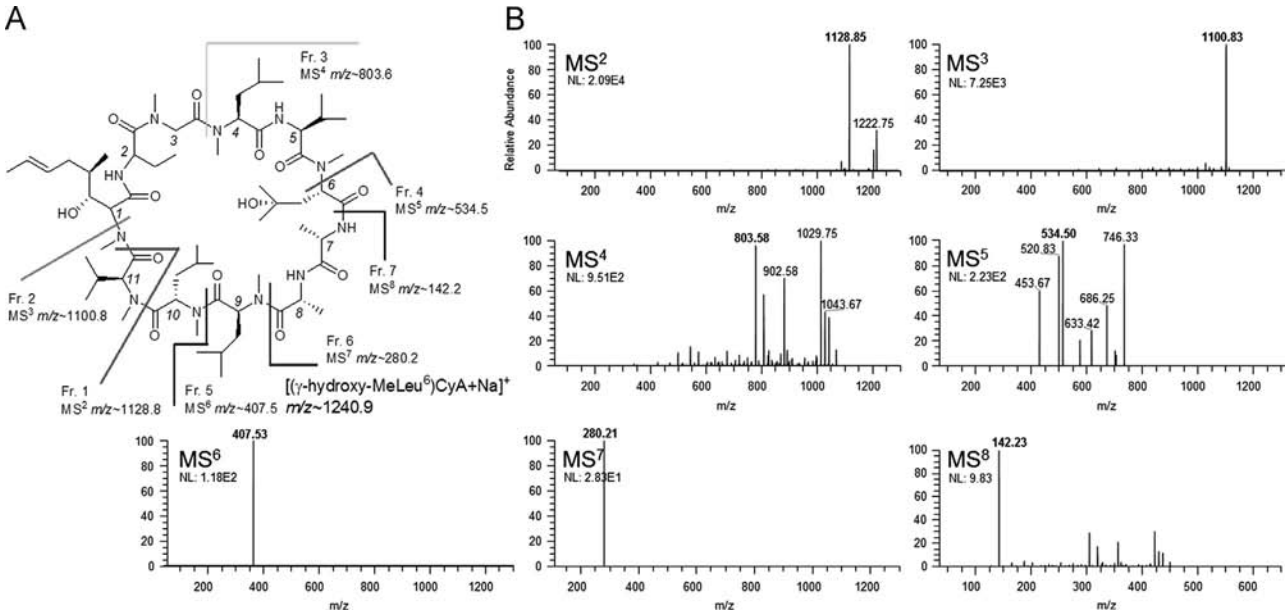

Fig. 5. Assignment of (A) fragmentation patterns of a bio-converted CyA analog, (γ-hydroxy-MeLeu^6)CyA (known as a human CYP metabolite AM6) and (B) its CID product ion spectra obtained from MS^2 to MS^8. Fragment ion assignments are performed for all diagnostic ions labeled below in the product ion spectrum.

hydroxylation site of AM6 is identical to the analog C_1, and so, we assigned C_1 as a (γ-hydroxy-MeLeu^6)CyA or AM6 [23]. Although the microbial bio-transformed analog C_1 is not novel CyA analog, this is the first report on the structural identification of the CyA analog by employing HPLC–ESI–ion trap MS as the MS^n platform.

4. Conclusions

Using HPLC–ESI–ion trap-MS carried out under our chosen conditions, the characteristic fragmentation pathways, not only for CyA and CyC, but also a bio-converted CyA analog, were elucidated, and their structures were proposed based on the major fragment ions that were observed through multiple tandem MS analyses. MS^n acquisition was accomplished on an LCQ ion trap mass spectrometer equipped with an electrospray interface operated in the positive ion mode. This is the first time that an ion trap system has been used for the comparative characterization of structurally related cyclosporins. The MS^n platform presented here allowed for more rapid and detailed studies of the fragmentation behavior, by performing up to MS^8 experiments; MS^n spectra of the corresponding CyA and CyC sodium adducts could be used as an interpretative guide for the fragmentation spectra of unknown analogs. Thus, using the MS^n capability of the ion trap, the structures of the bio-converted CyA analog present in the bacterial fermentation could be characterized as (γ-hydroxy-MeLeu^6)CyA (known as AM6 in human CYP metabolites).

Acknowledgments

This work was supported by Basic Science Research Program (20122039424, 20131014230) through the National Research Foundation of Korea (NRF) and the Intelligent Synthetic Biology Center of Global Frontier Project (20128054880, 20110031961) funded by the Ministry of Science, ICT & Future Planning, and the grant (PJ0094834) founded by the Next-Generation BioGreen21 program, Rural Development Administration.

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