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Characterization and identification of pradimicin analogs from *Actinomadura hibisca* using liquid chromatography–tandem mass spectrometry

Je Won Park[a], Sung Ryeol Park[b], Ah Reum Han[c], Yeon Hee Ban[b], Young Ji Yoo[b], Eunji Kim[b], Beom Seok Kim[d], Jae Kyung Sohng[a], Yeo Joon Yoon[b,*]

[a] *Department of Pharmaceutical Engineering, Institute of Biomolecule Reconstruction, Sun Moon University, Chungnam, South Korea*
[b] *Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, South Korea*
[c] *Interdisciplinary Program of Biochemical Engineering and Biotechnology, Seoul National University, Seoul, South Korea*
[d] *Division of Biotechnology, College of Life Sciences & Biotechnology, Korea University, Seoul, South Korea*

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ABSTRACT

Microbial cultures produce complex and potentially interesting mixtures of biosynthetic intermediates and derivatives of metabolites. These mixtures' reliable identification is important and so too is the development of techniques for their analysis. Here, a simple and highly selective method of detecting the biosynthetic congeners involved in the pentangular polyphenol pradimicin (PR) pathway from *Actinomadura hibisca* fermentation was developed. Solid-phase extraction (SPE) cleanup using an OASIS HLB cartridge was a simple and reliable tool for the extraction of PRs from a fermentation broth. The separation of each natural PR analog – eluted with a gradient system of aqueous acetonitrile through a reversed-phase C_{18} column containing ammonium acetate and acetic acid as additives – allowed their simultaneous profiling. The combined use of SPE cleanup and chromatographic separation, coupled with electrospray ionization–tandem mass spectrometry (ESI–MS/MS) detection was demonstrated to be sufficiently accurate and reliable to analyze the natural PR analogs produced from *A. hibisca*. Ten natural PRs were identified: four alanine-containing (PRA, PRC, PRL, and PRB), two glycine-substituted (PRD and PRE), and four serine-substituted (PRFA-1, PRFA-2, PRFL, and PRFB). This report demonstrates the first use of both SPE cleanup and HPLC–ESI–MS/MS to profile a wide range of structurally closely related PRs in a bacterial fermentation broth.

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1. Introduction

Pradimicins in the culture broth of actinomycete *Actinomadura hibisca* are a series of pentangular polyphenols that display potent antifungal and antiviral activities [1–4]. Since the first isolation of pradimicin A (PRA) as a major product from a producing strain [5], there have been a number of reports on pradimicin (PR) congeners, which possess a benzo[α]naphthacene aglycone as a common aromatic polyketide backbone, but are distinctly decorated with diverse combinations of both amino acids – including D-alanine (Ala), D-glycine (Gly) or D-serine (Ser) – and sugar moieties, such as thomosamine (Tho), des-*N*-methyl-thomosamine (DMTho), xylose (Xyl) or glucose (Glu) (Fig. 1a) [6–10]. PRA contains Ala, Tho, and Xyl as the appendage scaffolds; pradimicin B (PRB) does not have Xyl; and pradimicin C (PRC) contains DMTho instead of Tho aminosugar. Pradimicin L (PRL) has a Glu moiety instead of Xyl; and pradimicins D (PRD) and E (PRE) are the respective Gly-substituted analogs of PRA and PRC, in which Ala is replaced by Gly; whilst pradimicins FA-1 (PRFA-1) and FA-2 (PRFA-2) are the respective Ser-substituted analogs of PRA and PRC. The remaining pradimicins FL (PRFL) and FB (PRFB) are the respective Ser-substituted analogs of PRL and PRB. These intricate structural features complicate their efficient and simple differentiation and characterization.

To date, PR analogs have been isolated from pilot-scale fermentations using flash chromatography and separately structurally elucidated by nuclear magnetic resonance (NMR) spectroscopy [6–10]. However, there has been no report of systematic HPLC profiling of PRs and their intermediates biosynthesized from *A. hibisca*. Biotechnological advances in the characterization and manipulation of particular microbial biosynthetic gene clusters have encouraged the exploration of various antibiotic biosyntheses [11,12]. The PR biosynthetic gene cluster has recently been sequenced, providing genetic information on PR biosynthesis [13–15]. Further detailed characterization of PR biosynthetic pathways and functional identification of the genes involved requires more efficient detection of the biosynthetic intermediates formed with the PR complexes. Therefore, the development of a metabolite profiling method which can reliably and simultaneously detect

* Corresponding author. Tel.: +82 2 3277 4446; fax: +82 2 3277 3419.
E-mail address: joonyoon@ewha.ac.kr (Y.J. Yoon).

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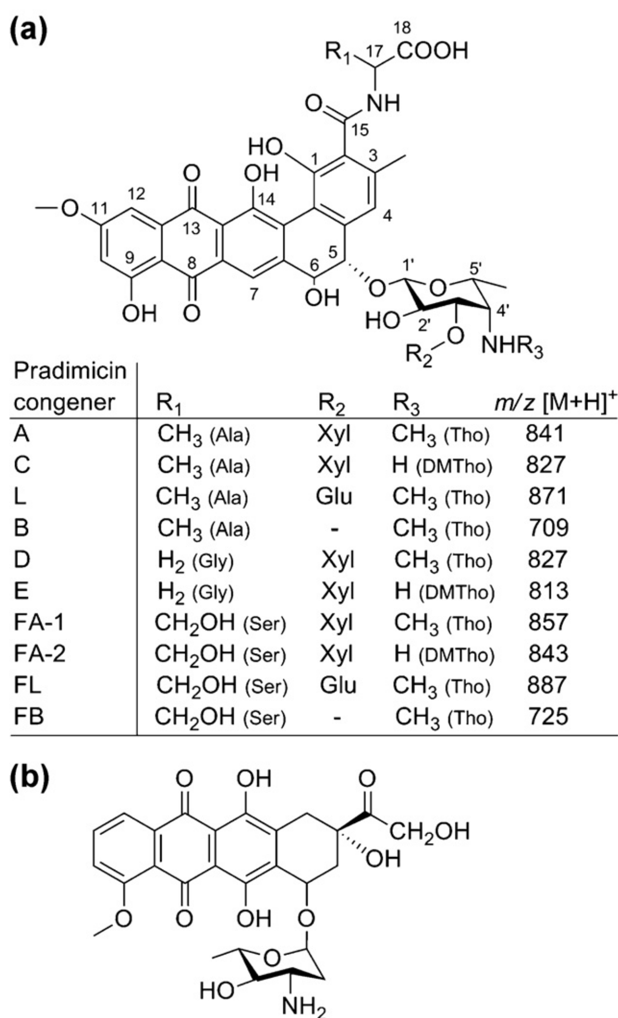

Pradimicin congener	R_1	R_2	R_3	m/z $[M+H]^+$
A	CH_3 (Ala)	Xyl	CH_3 (Tho)	841
C	CH_3 (Ala)	Xyl	H (DMTho)	827
L	CH_3 (Ala)	Glu	CH_3 (Tho)	871
B	CH_3 (Ala)	-	CH_3 (Tho)	709
D	H_2 (Gly)	Xyl	CH_3 (Tho)	827
E	H_2 (Gly)	Xyl	H (DMTho)	813
FA-1	CH_2OH (Ser)	Xyl	CH_3 (Tho)	857
FA-2	CH_2OH (Ser)	Xyl	H (DMTho)	843
FL	CH_2OH (Ser)	Glu	CH_3 (Tho)	887
FB	CH_2OH (Ser)	-	CH_3 (Tho)	725

Fig. 1. Chemical structures of (a) ten different pradimicins (PRs) found in *Actinomadura hibisca* fermentation broth and (b) doxorubicin used as an internal standard (IS) in this study. Ala: D-alanine; Gly: D-glycine; Ser: D-serine; Xyl: xylose; Glu: glucose; Tho: thomosamine; DMTho: des-*N*-methyl-thomosamine.

and identify the PR intermediates found in the culture of *A. hibisca* will be able to provide the significant details verifying the gene-to-metabolite network. This method is likely to involve electrospray ionization–tandem mass spectrometry (ESI–MS/MS), because it supplies definite chemical and structural evidence which can identify PRs' molecular masses and their distinct fragmentation patterns [16–18].

This study develops and evaluates an integrated analytical protocol comprising solid-phase extraction (SPE) cleanup and HPLC–ESI–MS/MS to detect and selectively identify a range of PRs produced from *A. hibisca* fermentation. PRs' profiles from the producing strain can be useful for discerning their biosynthetic pathways in combination with functional genomic approaches, and be a practical index to further studies, such as pathway engineering and combinatorial biosyntheses for generating new PR antibiotics with enhanced biological activities.

2. Experimental

2.1. Reagents and bacterial culture conditions

Doxorubicin hydrochloride as internal standard (IS) was obtained from GeneChem Inc. (Daejeon, Republic of Korea) (Fig. 1b), MS-compatible ammonium acetate and formic acid were from Fluka Chemie GmbH (Buchs, Switzerland), and HPLC-grade methanol, acetonitrile, glacial acetic acid, and water were from J.T. Baker (Philipsburg, NJ, USA). The culture medium components, yeast and malt extracts were acquired from BD (Sparks, MD, USA). Concentrated hydrochloride and glucose were of reagent grade. The SPE cartridges, including OASIS HLB ($3\,cm^3$/60 mg) and Sep-pak C18, and vacuum manifold were obtained from Waters (Milford, MA, USA), the AccuBOND C_{18}, Bond Elut C18, and Supelclean ENVI-18 SPE cartridges were purchased from Agilent (Palo Alto, CA, USA), Varian (Harbor City, CA, USA), and Supelco (Bellefonte, PA, USA), respectively. A stock IS solution ($10\,\mu g\,mL^{-1}$) was prepared in methanol, stored in an amber polypropylene microtube (Axygen Scientific, Union City, CA, USA) at −20 °C, The PR producer *A. hibisca* ATCC 53557, obtained from American Type Culture Collection (Rockville, MD, USA), has been described previously [14]. This strain was grown for one week at 28 C in 50 mL ISP2 medium (0.4% yeast extract, 1% malt extract, and 0.4% glucose; pH 7.0) in 500 mL baffled Erlenmeyer flasks on a rotary shaker at 180 rpm. The entire culture broth was subjected to further PR analysis. All experiments were carried out in triplicate.

2.2. Authentic PRA preparation

We isolated and purified PRA as an authentic standard directly from the fermentation broth of *A. hibisca* ATCC 53557 because PRs are not commercially available. Preparative HPLC was performed with a Spherisorb S5 ODS2 (Waters, 3.5 μm, 250 mm × 20 mm) semi-prep column eluted with 80% aqueous acetonitrile (v/v) containing 0.1% formic acid at a flow rate of $10\,mL\,min^{-1}$ over a period of 120 min, and the chromatogram was monitored by absorbance at 230 nm. The selected peak appeared at retention time ranging 37.7–39.1 min was pooled and freeze-dried, yielding PRA as a red amorphous powder (0.71 mg; ~94% pure by HPLC). ^1H NMR spectra were acquired by using a Varian INOVA 500 spectrometer (Varian, Inc., Palo Alto, CA, USA), and chemical shifts of PRA were assigned as follows; ^1H NMR (500 MHz, DMSO-d_6): δ 1.18 (d, 5′-CH_3), 1.43 (d, 17-CH_3), 2.48 (s, 3-CH_3), 2.61 (s, 4′-NCH_3), 3.04–3.73 (m, 4′, 2″, 3″, 4″-H), 3.75 (m, 2′-H), 3.79 (dd, 5″-H), 3.87 (q, 5′-H), 3.91 (m, 3′-H), 3.99 (s, 11-OCH_3), 4.39 (q, 17-H), 4.44 (d, 1″-H), 4.59 (d, 5-H), 5.03 (d, 6-H), 5.21 (d, 1′-H), 6.83 (d, 10-H), 6.99 (s, 4-H), 7.03 (d, 12-H), 7.34 (s, 7-H), 8.45 (d, 16-NH). These obtained data were in good agreement with those reported in the previous literatures [6,7]. The purified PRA stock solution ($10\,\mu g\,mL^{-1}$) was prepared in methanol, and serially diluted to make working standard solutions ($0.1–1.0\,\mu g\,mL^{-1}$), which are spiked into the blank fermentation media.

2.3. Extraction and cleanup

The extraction and cleanup of the PR congeners from the *A. hibisca* cultures before HPLC–ESI–MS/MS analysis involved organic extraction with methanol and SPE cleanup using an Oasis HLB cartridge (Waters). The whole cultures which were supplemented with IS ($10\,ng\,mL^{-1}$) were mixed with equal volumes of methanol, and agitated for 1 h on a laboratory rocker prior to centrifugation at 20,000 × *g* for 10 min at 4 °C. A 20 mL aliquot of the supernatant was subsequently loaded onto an Oasis HLB cartridge, previously conditioned with 3 mL methanol followed by 3 mL 50% (v/v) aqueous methanol. The cartridge was washed with 3 mL 50% aqueous methanol and air-dried under reduced pressure for approximately 30 s. The bound PRs of interest were eluted twice with 0.75 mL 5% (v/v) methanolic formic acid, and evaporated to dryness at room temperature by vacuum centrifugation, and then stored in a freezer. For analyses, the desired residue was reconstituted to 50 μL with methanol. Ten μL of this solution, corresponding to a culture volume of 4 mL, was subjected to HPLC–ESI–MS/MS anal-

Table 1
Accuracy and precision of the analysis of authentic pradimicin A spiked into a blank fermentation medium.

Concentration ($ng\,mL^{-1}$)		RSD (%)	Accuracy (%)
Added	Found (mean ± SD)		
Pradimicin A (n=15)			
Intra-day			
2.00	01.76 ± 0.11	5.5	88.0
10.00	09.28 ± 0.46	4.6	92.8
20.00	18.37 ± 1.07	5.4	91.8
Inter-day			
2.00	01.79 ± 0.11	5.5	89.5
10.00	09.22 ± 0.55	5.5	92.2
20.00	18.29 ± 1.19	5.9	91.4

Table 2
Chromatographic, ESI–MS/MS transitional details and the qualitative analysis of pradimicin profiles found in the culture of *Actinomadura hibisca* ATCC 53557.

Pradimicin	Retention time (min)	Mass transition (*m/z*)	Relative content (%)
PRA	21.8	841 > 292	63.9
PRC	29.6	827 > 278	13.6
PRL	26.2	871 > 322	0.7
PRB	31.2	709 > 160	0.9
PRD	22.5	827 > 292	0.6
PRE	24.7	813 > 278	6.5
PRFA-1	46.7	857 > 292	5.7
PRFA-2	35.6	843 > 278	4.3
PRFL	34.4	887 > 322	0.6
PRFB	41.7	725 > 160	3.2

ysis. The recoveries of the authentic PRA (final $10\,ng\,mL^{-1}$) spiked into a blank fermentation medium were determined to check the performance of the SPE cleanup as well as matrix effect of the media on the isolation of the PR congeners during extraction and cleanup. The spiked PRA was extracted as described above and analyzed further by HPLC–ESI–MS/MS. The percentage recoveries of PRA were calculated, using three replicates, from a comparison of the chromatographic peak areas obtained from both spiked blank samples with those from the PRA working solutions. To evaluate the SPE cleanup procedure, the intra- and inter-day precision and accuracy were examined by analyzing PRA spiked (2, 10, and $20\,ng\,mL^{-1}$) into a blank fermentation medium with five replicates on three separate days, and are presented as the relative standard deviations (RSD) (Table 1).

2.4. HPLC–ESI–MS/MS analysis

Analytical HPLC–ESI–MS/MS was performed on a Waters/Micromass Quattro micro/triple quadrupole MS interface using a Synergi Polar-RP column (Phenomenex, Torrance, CA, USA; 4.0 μm, 150 mm × 4.6 mm). Analytes were eluted at a flow rate of $250\,\mu L\,min^{-1}$ using a gradient of water with 5 mM (w/v) ammonium acetate and 0.05% (v/v) acetic acid (solution A) and 80% (v/v) aqueous acetonitrile with the same concentrations of additive (solution B) at 20–70% B for 25 min, to 80% B for 15 min, maintained at 80% B for 10 min, and then to 20% B for another 10 min for column re-equilibration. The column effluent was directed to the ESI–MS/MS, which was operated in positive ion mode without splitting. The instrument was tuned by the direct infusion of authentic PRA stock solution ($10\,\mu g\,mL^{-1}$) in the ion source at $50\,\mu L\,min^{-1}$. The ESI–MS/MS system was optimized based on maximum generation, first of the protonated molecular ion (precursor), and then of the corresponding fragment (product) ion. The following calibration parameters were used: *m/z* 130–900 scan ranges; 5.0 kV capillary voltage; 33 V cone voltage; 130 °C source temperature; 250 °C desolvation temperature; $550\,L\,h^{-1}$ desolvation gas flow rate; and $55\,L\,h^{-1}$ cone gas flow rate. The collision energy in MS/MS mode, concurring with the full argon-induced fragmentation of the parent ions, was 1.5 V (pressure reading 8.05×10^{-6} mbar). The molecular structures and the proposed fragmentation patterns of the PR biosynthetic intermediates produced during *A. hibisca* fermentation, which were obtained under the above-mentioned conditions, are illustrated (Fig. 2). In order to evaluate the sensitivity and feasibility of this analytical profiling technique for the quantitative PR detection, authentic PRA spiked into a blank fermentation medium was quantified by MS/MS in multiple reactions monitoring (MRM) mode.

This was done by using two mass ions (listed in Table 2) set to trace a transition of the protonated precursor to the product ion typical to the selected analytes: IS, *m/z* 544 > 130; PRA, *m/z* 841 > 292; PRC, *m/z* 827 > 278; PRL, *m/z* 871 > 322; PRB, *m/z* 709 > 160; PRD, *m/z* 827 > 292; PRE, *m/z* 813 > 278; PRFA-1, *m/z* 857 > 292; PRFA-2, *m/z* 843 > 278; PRFL, *m/z* 887 > 322; PRFB, *m/z* 725 > 160.

3. Results and discussion

3.1. Setup the analytical protocol

Prior to establishing a complete method, IS and authentic PRA were used as a mimic and a reference PR, respectively. The recoveries of authentic PRA spiked into the blank fermentation media were checked at the SPE cleanup step to simplify the analytical scheme and to obtain extracts more quickly. The conditions of HPLC–ESI–MS/MS were set both to separate the PRs and to provide molecular information of each PR biosynthetic intermediate. The fermentation media supplemented with IS and authentic PRA at $10\,ng\,mL^{-1}$, respectively, were mixed with an equal volume of methanol and loaded on to a variety of SPE cartridges from the different suppliers described in Section 2.1. The resulting extract was then subjected to HPLC–ESI–MS/MS analysis. The highest mean recovery of authentic PRA spiked into a fermentation medium (93 ± 3%) was observed on the OASIS HLB, and IS was also recoverable enough to detect (89 ± 4%) in the same cartridge. The other reversed-phase C_{18} cartridges gave significantly lower extraction recoveries of authentic PRA ranging from 48 to 76%. Moreover, it was observed that the extracts obtained from the use of the C_{18}-based cartridges brought out a very deep color, and the chromatogram of the extracts showed a number of messy peaks that could seriously interfere with the PR analysis. The presence of these endogenous interferences would also lead to the suppression of the ionization of PR in the ESI–MS/MS detection procedure. On the other hand, the resulting extracts from the OASIS HLB cartridge was visually much cleaner than those from the others, that is likely to aid subsequent ESI–MS/MS analysis. This difference was probably caused by the adsorbing strengths of PR onto the cartridges; resins charged in the OASIS HLB column are known to possess a balanced hydrophilic–lipophilic interface. In addition, no matrix effect on the detection of IS spiked into the cultures was observed (retention time shift and non-symmetric peak) in extracts obtained when OASIS HLB cartridges were used. Therefore, single use of OASIS HLB cleanup appears to be sufficient to recover PR as well as IS, demonstrating this SPE cleanup as a simple and rapid technique to isolate the glycosylated polyphenols from complicated microbial resources.

The mass transitions (*m/z* precursor > *m/z* product ion) of IS and authentic PRA in the positive mode of HPLC–ESI–MS/MS were 544 > 130 and 841 > 292, respectively. Protonated molecular ions $[M+H]^+$ generated a tandem mass spectrum with diagnostic product ions. As expected from previous studies, glycosidic bond cleavage was the main fragmentation pattern; a product

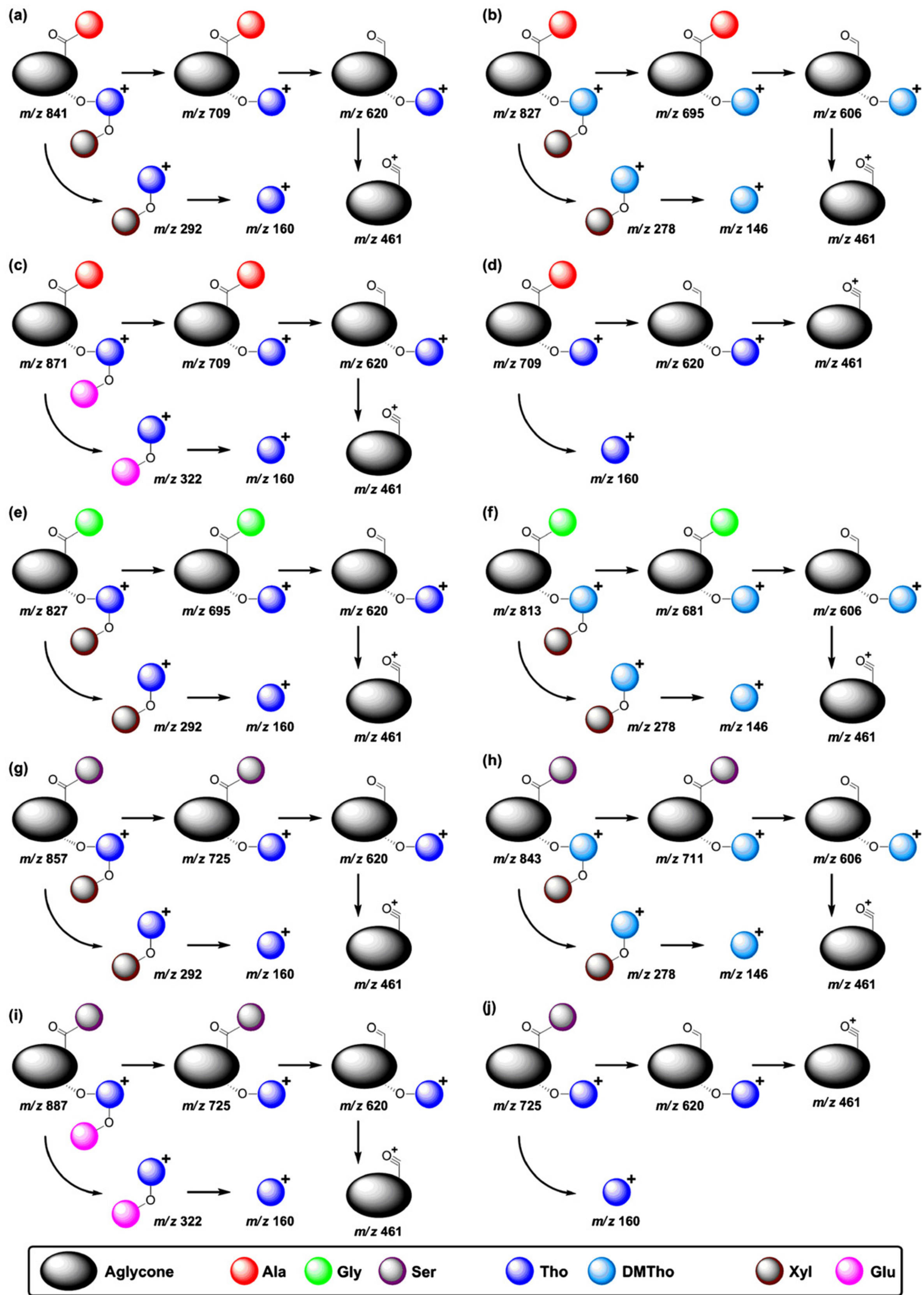

Fig. 2. The proposed ESI–MS/MS fragmentation pathways of (a) pradimicin A (PRA), (b) PRC, (c) PRL, (d) PRB, (e) PRD, (f) PRE, (g) PRFA-1, (h) PRFA-2, (i) PRFL and (j) PRFB. Shaded oval and circles in the block symbolize the several moieties appended to the PRs.

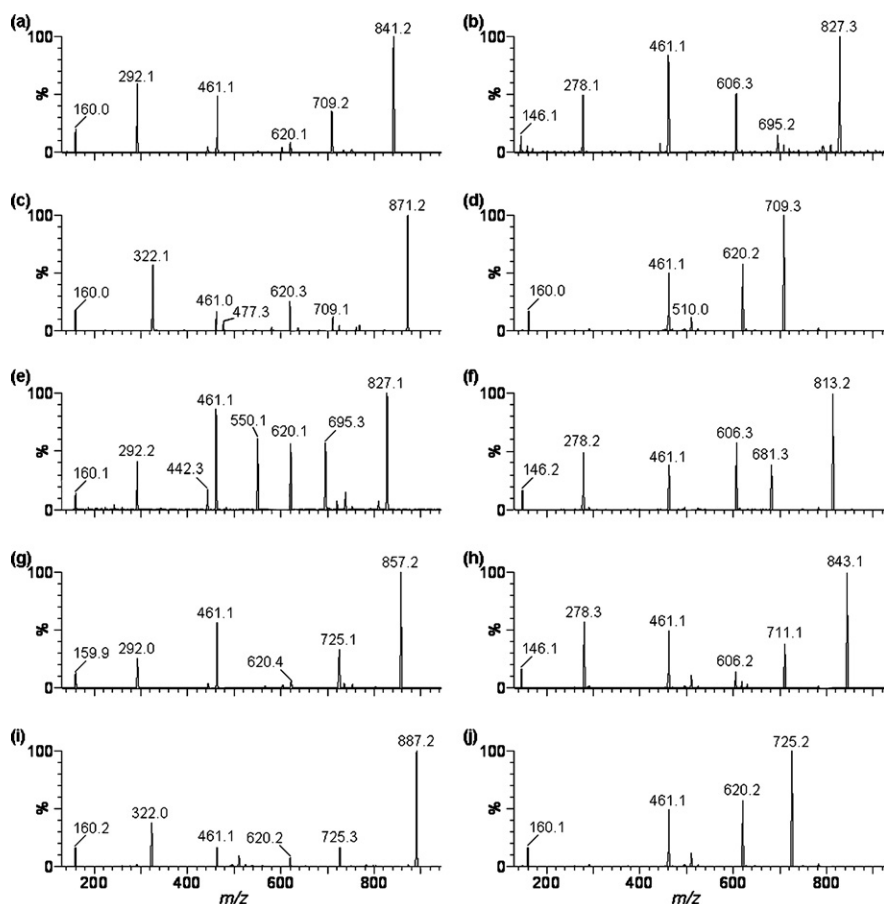

Fig. 3. Representative MS/MS spectra of (a) pradimicin A (PRA), (b) PRC, (c) PRL, (d) PRB, (e) PRD, (f) PRE, (g) PRFA-1, (h) PRFA-2, (i) PRFL and (j) PRFB detected in the *Actinomadura hibisca* fermentation.

ion observed for IS at *m/z* 130 corresponds to a protonated aminosugar moiety L-daunosamine [19–21], whereas a fragment ion for authentic PRA at *m/z* 292 matches to a protonated Xyl-attached Tho. Most therapeutic drug monitoring studies of MS/MS transitions of authentic IS using ESI-interfaced ion trap- and tandem-mass spectrometers show that the dominant ion was assigned at *m/z* 130, identical to the results of this work [20,21]. Therefore, the appearance of specific fragmented ions can be used to identify the analyte; and the characteristic mass transition between the protonated precursor and the product ion can be used to quantify and complete the metabolite profiling using HPLC–ESI–MS/MS in MRM mode. The detection limit (LOD) of authentic PRA obtained from serial dilution of working solution at a signal-to-noise ratio of 15:1 was determined as 6.6 ng per an injection (10 μL), corresponding to 660 ng mL^{-1}. There was a good linear correlation ($r^2 > 0.999$) between the amount of authentic PRA spiked into blank fermentation media (2–40 ng mL^{-1}) and the ESI–MS/MS response (estimated as peak height). The limit of quantification (LOQ) was determined as the lowest concentration of authentic PRA that could be quantified when spiked into the fermentation medium. Based on the mean recovery (88 ± 5%) of authentic PRA spiked at LOD level and the concentration factor (400; the ratio of final concentrated volume-to-fermentation medium volume, 10 μL to 4 mL), LOQ was estimated as approximately 1.9 ng mL^{-1}. Intra- and inter-day precision and accuracy using fermentation media spiked with authentic PRA at three different levels (2, 10, and 20 ng mL^{-1}) were examined. All spiked samples were extracted as described in Section 2.3 and analyzed further by HPLC–ESI–MS/MS. The precision (RSD) were all below 6%, and the intra-day and inter-day accuracy ranged from 88.0 to 92.8% and 89.5 to 92.2%, respectively (Table 1). Therefore, these data confirm that the combined use of the simple and rapid OASIS HLB SPE cleanup and the sensitive HPLC–ESI–MS/MS detection at nanogram level is likely to be sufficiently precise and reproducible for the profiling of PRs produced from a fermentation broth.

3.2. Analytical profiling and characterization of pradimicin congeners from the A. hibisca strain

The above-described method was used to detect the PRs produced by an *A. hibisca* strain. A number of peaks were detected based on the protonated molecular ions of the PRs listed in Fig. 1a whose presence was previously established from earlier specialized studies of large-scale fermentation, flash chromatographic isolation, and NMR [5–10,22]. Profiling using HPLC–ESI–MS/MS appears to be a simple and sensitive alternative to conventional large-scale fermentation and isolation for identifying each PR without prior chromatographic separation if the PR structures are predictable by MS/MS fragmentation patterns or are already known. The ten natural PR analogs detected in the fermentation broth are listed by order of chromatographic elution: PRA (*m/z* 841), PRD (*m/z* 827), PRE (*m/z* 813), PRL (*m/z* 871), PRC (*m/z* 827), PRB (*m/z* 709), PRFL (*m/z* 887), PRFA-2 (*m/z* 843), PRFB (*m/z* 725), and PRFA-1 (*m/z* 857). The identity of each analog was characterized by comparing the proposed fragmentation patterns shown in Fig. 2 with the product ions in the ESI–MS/MS spectra of Fig. 3. A qualitative comparison of the abundances of PRs detected during fermentation was carried out using MRM mode, which monitored the unique mass transition specific to each PR (Fig. 4).

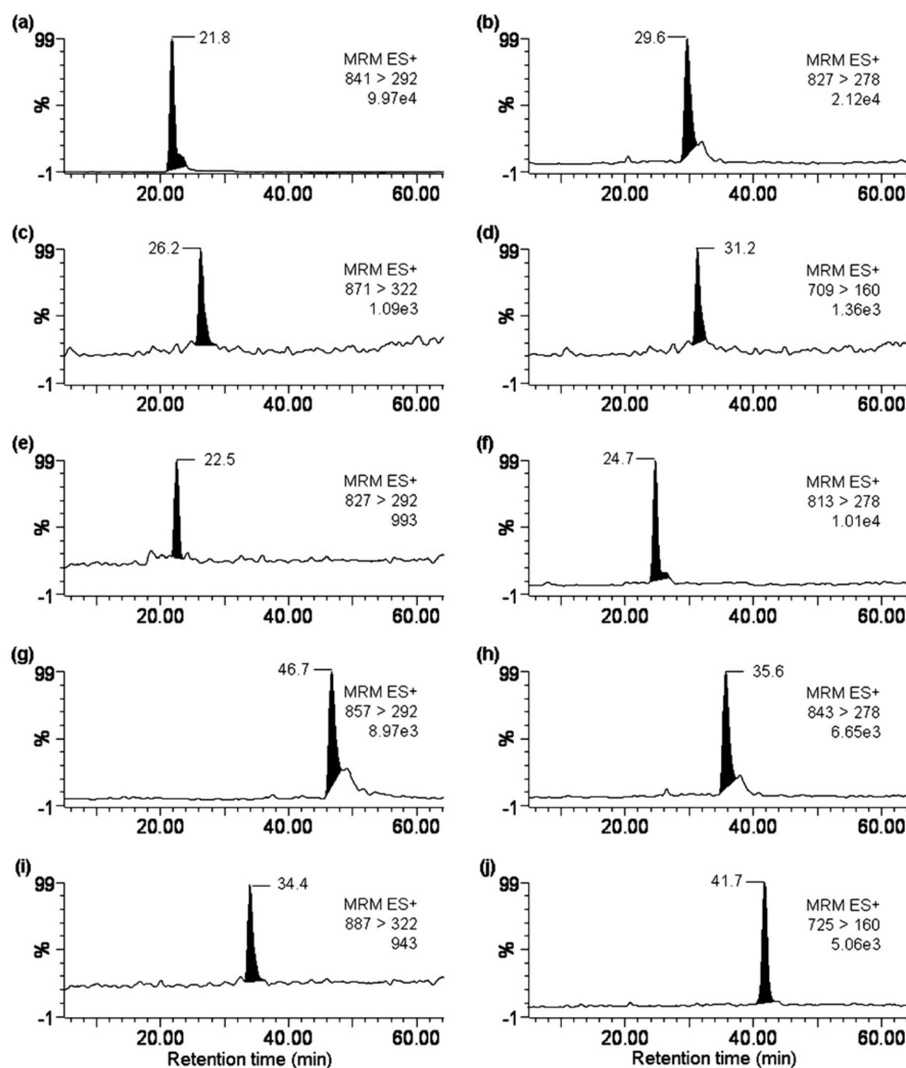

Fig. 4. Representative multiple reactions monitoring (MRM) chromatograms of (a) pradimicin A (PRA), (b) PRC, (c) PRL, (d) PRB, (e) PRD, (f) PRE, (g) PRFA-1, (h) PRFA-2, (i) PRFL and (j) PRFB detected in the *Actinomadura hibisca* fermentation.

The first peak selected by the HPLC–ESI–MS scan at *m/z* 841 shown at a retention time (Rt) of 21.8 min is of a protonated molecular ion of the main pradimicin metabolite PRA. It was then subjected to ESI–MS/MS analysis (Table 2 and Fig. 4a) which yielded several product ions at *m/z* 709, 620, 461, 292 and 160 (Fig. 3a), that were identical to the suggested fragmentation pattern of PRA (Fig. 2a). The loss of Xyl, the subsequent loss of Ala moiety, and the resulting aglycone from the PRA molecule correspond to fragment ions at *m/z* 709, 620 and 461, respectively, whereas the existence of Xyl-attached Tho and Tho correspond to fragment ions at *m/z* 292 and 160, respectively. These indicate that, without exception, the fragmentation of PR occurs at glycosidic linkages and the amide bond. In particular, a difference of 89 Da between *m/z* 709 and 620 could be used as one of the diagnostic fragmentation patterns for Ala-containing PR congeners. There are three additional Ala-containing PRs – PRB, PRC and PRL – present in *A. hibisca* fermentation [6,9]. By selection of their protonated molecular ions at *m/z* 709, 827 and 871, four peaks with Rt of 22.5, 26.2, 29.6 and 31.2 min were subjected to HPLC–ESI–MS/MS analysis (Table 2 and Fig. 3). The peak with Rt 29.6 min captured by *m/z* 827 was presumed to be of PRC, composed of the Ala and Xyl moieties as PRA, but containing DMTho instead of the Tho in PRA. The MS/MS spectrum of this molecular ion at *m/z* 827 illustrates the presence of the predicted fragment ions at *m/z* 695, 606, 461, 278 and 146 (Figs. 2b and 3b). Compared with the above MS/MS spectrum of PRA, the loss of 14 Da on the fragments specific to amino sugars (*m/z* 146 and 278) due to the demethylation of Tho moieties and the lack of change of the aglycone fragment ion at *m/z* 461 suggest that this peak is of PRC. Also, the existence of a difference of 89 Da, indicative of Ala-containing PRs, confirmed this peak as PRC. Other peaks with Rt 26.2 and 31.2 min, captured by *m/z* 871 and 709, separately, were respectively identified as PRL and PRB (Fig. 3c and d). The MS/MS spectrum of an earlier peak showed the fragment ions at *m/z* 160 and 322 – specific to Tho and the disaccharide Glu-attached to Tho on the parent molecule, respectively – whereas the MS/MS spectrum of the later peak generated the sole amino sugar fragment at *m/z* 160 corresponding to Tho monosaccharide on the aglycone molecule. Furthermore, the MS/MS spectra of the above two peaks had a difference of 89 Da (from *m/z* 709 to 620), an index for the Ala-containing PRs. The fragment ions of both molecules produced by MS/MS analysis were well fitted to fragmentation pathways proposed for PRL and PRB (Fig. 2c and d).

When captured by *m/z* 827, two peaks with Rt 22.5 and 29.6 min were monitored, with the latter being identified as PRC (Table 2). The MS/MS spectrum of the earlier peak showed product ions quite different from those of PRC: *m/z* 160 and 292, corresponding to Tho and Xyl-attached Tho, as well as a difference of 75 Da (from *m/z* 695 to 620), which could be due to the substitution of Ala with Gly moiety on the PRs (Figs. 2e and 3e). Therefore, the Rt 22.5 min

peak appears to be from one of the Gly-containing PR analogs, PRD (Table 2). Another Gly-containing PR, PRE, has been reported present in *A. hibisca* fermentation [7]. The peak selected by a protonated molecular ion of PRE at *m/z* 813 was detected with an Rt of 24.7 min (Table 2). MS/MS analysis of the above peak produced fragment ions at *m/z* 146 and 278 specific to DMTho and Xyl-attached DMTho moiety, respectively, as well as a 75 Da difference (from *m/z* 681 to 606) characteristic of a Gly moiety attached to aglycone through an amide bond (Figs. 2f and 3f), thereby verifying the identity of PRE.

Based on previous reports of the isolation of the four natural PRs from *A. hibisca* strain (PRFA-1, PRFA-2, PRFL and PRFB) in which Ser replaces Ala or Gly [8–10], HPLC–ESI–MS scans selected *m/z* 887, 843, 725 and 857, separately; thus four peaks of Rt 34.4, 35.6, 41.7 and 46.7 min were resolved (Table 2). MS/MS analysis of the peak captured by *m/z* 857, corresponding to a protonated molecular ion of PRFA-1 (Figs. 2g and 3g), indicated that some fragment ions at *m/z* 160 and 292, representing the presence of Xyl-attached Tho disaccharide moiety, as well as a difference of 105 Da (from *m/z* 725 to 620) were caused by the appendage of Ser on to the aglycone molecule, distinguishing this peak as one of the Ser-containing PR analog, PRFA-1. Another peak captured by *m/z* 843 was traced at a Rt of 35.6 min, and its MS/MS spectrum matched the proposed fragmentation pathway of PRFA-2 (Figs. 2h, 3h and 4h): fragment ions at *m/z* 146 and 278 indicate the glycosidic bond of Xyl-attached DMTho on the aglycone, compared with those of PRFA-1, and also a 105 Da difference which is indicative of the presence of Ser instead of Ala or Gly both suggest structural features of PRFA-2, in which a DMTho aminosugar moiety substitutes the Tho of PRFA-1. The two remaining peaks (Rts at 34.4 and 41.7 min) captured by *m/z* 887 and 725 are of the protonated molecular ions of PRFL and PRFB, respectively, and were subjected to MS/MS analyses (Table 2 and Fig. 3i and j). The earlier peak showed two product ions at *m/z* 160 and 322 representing the presence of Glu-attached Tho as in the case of PRL, whereas the MS/MS spectrum of the latter peak indicated the appendage of only a Tho sugar moiety (*m/z* 160) to the aglycone, as an analog to that of PRB (Figs. 2 and 3). The two molecules have also the 105 Da differences (from *m/z* 725 to 620) as the mass transition diagnostic for Ser-containing PRs, thus supporting their respective identifications as PRFL and PRFB.

Ten PR natural analogs were profiled, and their product ion patterns determined by HPLC–ESI–MS/MS were in good agreement with the proposed fragmentation pathways (Figs. 2 and 3). All the PRs described and identified in this study have been previously characterized. However, this is the first report of the simple and highly selective HPLC–ESI–MS/MS characterization of a wide range of natural PRs from a bacterial fermentation broth, without the need for time-consuming isolation and NMR spectroscopy. The ESI–MS/MS fragmentation patterns of the PRs suggest that they all contain the common product ion at *m/z* 461, which corresponds to aglycone without attached amino acids or sugars (Figs. 2 and 3). This appears to be a conserved product ion implicated in the PR biosynthetic pathway, and hence precursor ion mapping using this typical product ion (*m/z* 461) can provide useful information about PRs produced from wild-type *A. hibisca* or recombinant strains.

In order to demonstrate the feasibility and selectivity of PR detection using this profiling system, a qualitative abundance comparison of the PRs detected in the fermentation was made based on the relative heights of peaks monitored by MRM mode set at ten different mass transitions specific to the above natural PR analogs, listed in the order of relative content of PR in the fermentation broth (Fig. 4 and Table 2): PRA (63.9%), PRC (13.6%), PRE (6.5%), PRFA-1 (5.7%), PRFA-2 (4.3%), PRFB (3.2%), PRB (0.9%), PRL (0.7%), PRD (0.6%) and PRFL (∼0.6%). Previous quantitative analyses of PRs isolated from pilot-scale fermentations involved stepwise elution using a preparative reversed-phase resin [6,10,22]. They report that the major components were mixtures of PRA and PRC, constituting about 75% by weight of the separated PRs. These are in accordance with the qualitative data on the PRs reported here: PRA and PRC content was approximately 77%. Therefore, the method developed in this study for the profiling of PR in *A. hibisca* fermentation cultures appears to be a simple and sufficiently selective alternative to conventional large-scale fermentation and isolation processes. An examination of analytical profiles from a genetically engineered *A. hibisca* strain would be a helpful future study to further understanding of PR biosynthetic pathways [14], which are still yet not fully characterized. It may also be useful for unveiling the function of previously uncharacterized biosynthetic genes in the PR biosynthetic gene clusters.

4. Conclusions

The simultaneous determination of pradimicins (PRs) in *A. hibisca* fermentation was achieved through a novel integrated PR profiling technique comprising simple cleanup and resolvable separation. These two steps were respectively carried out using an OASIS HLB SPE cartridge and reversed-phase chromatography, without requiring any of the laborious procedures usually demanded by this type of analysis. The selective, reliable and facile identification of a total of ten PR biosynthetic intermediates was achieved by HPLC–ESI–MS/MS. This method demonstrated highly selective profiling of PRs produced in the *A. hibisca* culture, and is promising for examining omics-based studies, such as gene-to-metabolite networks for PR biosynthesis in microbial fermentation and for exploring microbes' metabolic diversity.

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