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Probing the in vivo biosynthesis of scytonemin, a cyanobacterial ultraviolet radiation sunscreen, through small scale stable isotope incubation studies and MALDI-TOF mass spectrometry

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ABSTRACT

Scytonemin is a dimeric indole phenolic pigment found in the sheaths of many cyanobacteria. This pigment absorbs UV radiation protecting subtending cyanobacterial cells from harmful effects. Based on scytonemin's unique chemical structure, the pathway to its biosynthesis is uncertain, thus motivating the current investigation. Herein, we report the incorporation of both tyrosine and tryptophan into scytonemin, and provide in vivo data supporting the tryptophan origin of the ketone carbon involved in the condensation of the two biosynthetic precursors. This study also reports on the new use of a small-scale, MALDI-TOF mass spectrometry technique to monitor the incorporation of isotopically labeled tyrosine during scytonemin biosynthesis.

1. Introduction

Scytonemin, a dimeric indole phenolic pigment, is found in the sheaths of many species of cyanobacteria and possesses powerful ultraviolet (UV) radiation-absorbing properties.[1,2] Its in vivo absorption in the UV-A range (λ_{max} = 370 nm) allows cyanobacteria to survive in environments with high sun exposure without negatively impacting photosynthesis.[3] Scytonemin's interesting chemical structure and ecological role as a sunscreen are complemented by its potentially valuable pharmaceutical role as a modulator of cell cycle control and inflammation.[4,5]

Scytonemin's unique dimeric structure, ecological importance and novel pharmaceutical activity have motivated considerable interest in its biosynthesis. In 2007, Soule et al. identified a biosynthetic gene cluster involved in scytonemin production in *Nostoc punctiforme* ATCC 29133 using in vivo transposon mutagenesis.[6] The mutation, found to inhibit scytonemin production, was located in a hypothetical protein (Np1273) associated with a gene cluster containing genes predicted to be involved in aromatic amino acid biosynthesis. The gene cluster was consistent with the original prediction that scytonemin was derived from indole and phenol-containing amino acids or their precursors.[2] The association of these genes as a cluster and their role in scytonemin biosynthesis was later supported by evidence for increased transcriptional expression of each gene in the cluster upon exposure of *N. punctiforme* ATCC 29133 to UV-A radiation.[6–8]

The availability of numerous cyanobacterial genomes has allowed identification of the scytonemin biosynthetic gene cluster across several cyanobacterial lineages.[7,9] Although some genetic variation exists between clusters, a majority of the genes were shown to have a high degree of amino acid sequence similarity, indicating that scytonemin biosynthesis evolved as a highly conserved process for cyanobacterial defense against UV radiation.[7,9] The identification of genes involved in the production of scytonemin has led to an improved understanding of the enzymatic mechanisms involved in the biosynthesis of this sunscreen pigment. One of these mechanisms involves the coupling of two aromatic amino acid precursors to form the scytonemin monomer. The indolic precursor is predicted to derive from the tryptophan branch of aromatic amino acid biosynthesis, while the phenolic portion may originate from hydroxyphenylpyruvate (HPP) created from prephenate using prephenate dehydrogenase (Np1269), an enzyme encoded in the scytonemin gene cluster. A recombinant Np1275 was shown to be involved in the oxidative deamination of tryptophan to form indole-3-pyruvic acid (IPA). When IPA and HPP were incubated with another gene product from the gene cluster, Np1276, this latter enzyme was shown to catalyze a selective

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acyloin reaction.[10] The product of this reaction was shown to be the substrate for an intermolecular attack by the indole ring followed by facile decarboxylation catalyzed by Np1274 to yield a cyclopentane.[11] Overall, these enzymatic reactions result in the formation of the tricyclic cyclopentylindole framework representing the monomeric unit of scytonemin.

However, two distinct pathways could result in this product, and these differ in the origin of the two carbons involved in the coupling of these precursors (Fig. 1). We reasoned that in vivo data derived from the incorporation of isotopically labeled aromatic amino acid derived substrates could lead to an understanding of the biogenesis of this carbon framework. In this study, we report the use of small-scale MALDI-TOF mass spectrometry to monitor the in vivo incorporation of isotopically labeled precursors during scytonemin biosynthesis. These results support previous in vitro studies of scytonemin biosynthesis by identifying both tyrosine and tryptophan as the in vivo precursors to scytonemin, and further reveal that the ketone carbon involved in the condensation of these two biosynthetic precursors is derived from the tryptophan subunit.

2. Results and discussion

The unique molecular scaffold of scytonemin contains both indolic and phenolic moieties, suggesting that they may derive from tryptophan and tyrosine, respectively. This hypothesis was examined by monitoring for incorporation of these aromatic amino acid-derived subunits using stable isotope enrichment studies. To initiate this investigation, ten different species of freshwater cyanobacteria were placed under UV irradiance to evaluate their ability to produce scytonemin under laboratory conditions. Of these, five survived the UV exposure treatment, including *Nostoc* sp. PCC7120, *Fischerella musicola*, *Lyngbya* sp., *Oscillatoria tenius*, and *Tolypothrix distorta*. These five species, as well as *N. punctiforme* ATCC 29133, were analyzed for scytonemin using MALDI-TOF mass spectrometry. *T. distorta* was identified as a scytonemin producer on the basis of a monoisotopic mass at *m/z* 546 and an isotopic ratio pattern matching a purified scytonemin standard. *T. distorta* was found to produce scytonemin in reliable quantities for analytical analyses, such as MALDI-TOF mass spectrometry, and therefore, was chosen for subsequent biosynthetic studies using stable isotope enrichments.

We envisioned the use of MALDI-TOF mass spectrometry for the small-scale analyses of UV-induced *T. distorta* to provide a quick and cost effective analysis of stable isotope incorporations in this organism. As a control, a single tuft (approximately 7 mg wet wt) from the *T. distorta* culture was exposed to UV radiation in the absence of isotopically labeled precursors in the media. MALDI-TOF revealed a monoisotopic mass at *m/z* 546 for scytonemin (Fig. 2A) with smaller peaks at *m/z* 547 and 548 representing incorporation of ^{13}C due to its natural abundance of 1.1%.[12] When a cultured tuft of *T. distorta* was analyzed after incubation with L-tyrosine-d_2 (tyr-d_2) and exposed to UV radiation, MALDI-TOF revealed isotopic mass peaks at $[M+2]^+$ *m/z* 548 and $[M+4]^+$ *m/z* 550, representing incorporation of one or two tyr-d_2 precursors into the scytonemin scaffold (Fig. 2B). The small scale MALDI-TOF results from *T. distorta* correlate with our large scale MS and ^1H NMR characterization of the incorporation of tyr-d_2 into scytonemin from *N. punctiforme* ATCC 29133 (Fig. 3). Advantages of the MALDI-TOF approach were that the analysis required only a very small amount of labeled precursor (<10 mg), minimal amounts of the cultured organism, and allowed for the entire experiment to be rapidly completed (<5 days) compared to 500 mg of labeled precursor, 3 L of cultured organism and a much longer experimental period (weeks) for a large scale NMR study.

The relative sensitivity of MALDI-TOF and the short incubation time prior to analysis also allowed us to overcome problems due to the inherent photochemical instability of tryptophan that we

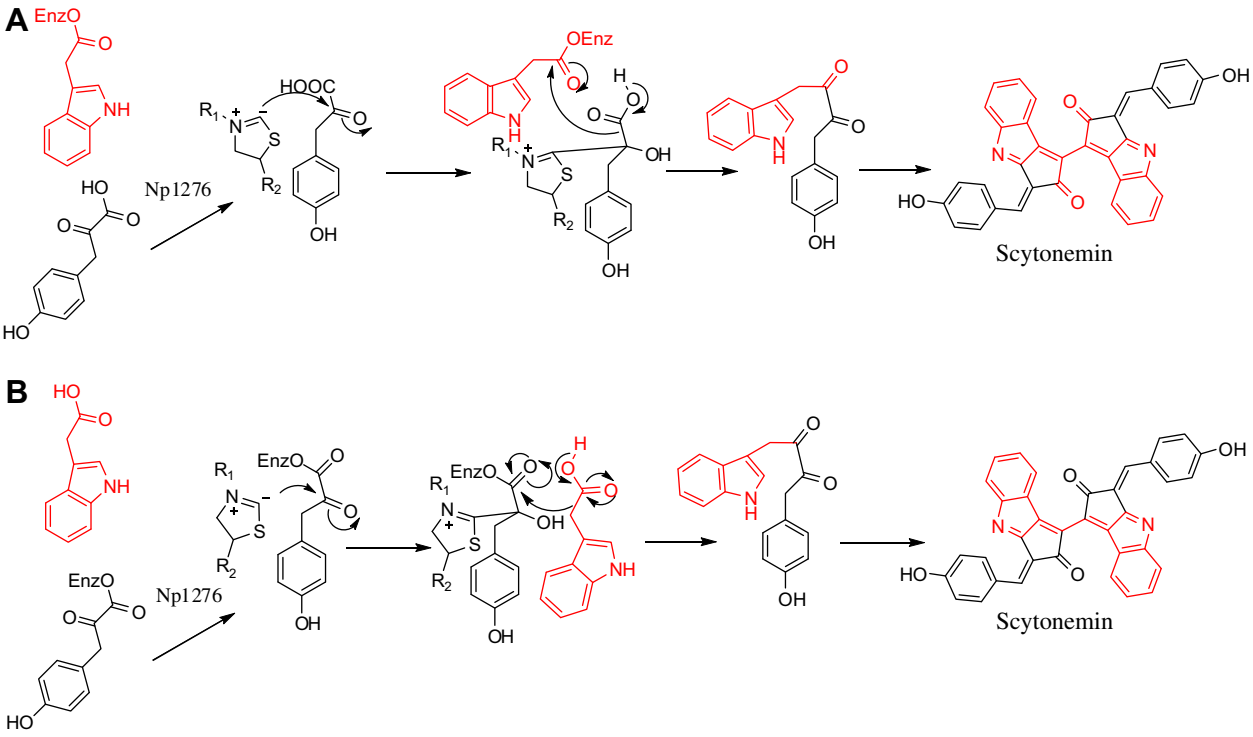

Figure 1. Two potential mechanisms for the formation of a diketone precursor shown to form during scytonemin biosynthesis.[10] (A) Scheme showing ketone carbon of scytonemin deriving from indole-3-acetic acid (IAA) and (B) scheme showing ketone carbon of scytonemin deriving from 4-hydroxyphenylpyruvate (HPP). IAA is drawn in red and HPP is drawn in black.

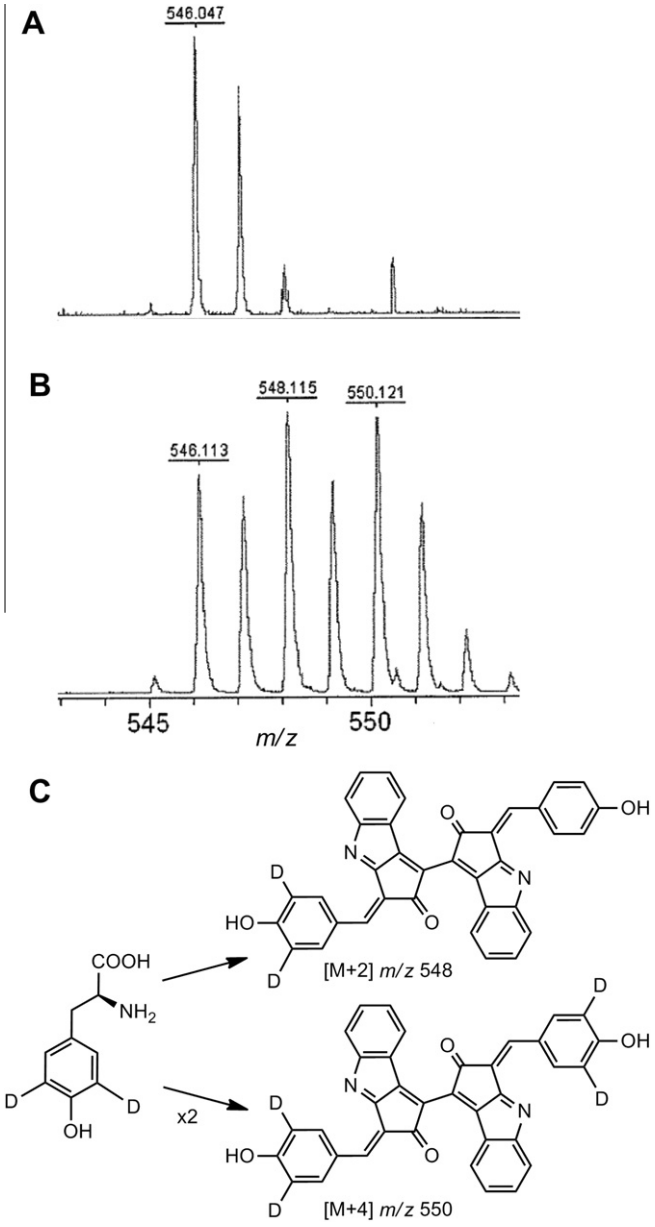

Figure 2. MALDI-TOF spectra of *T. distorta* extract after incubation with L-tyrosine-3,5-d_2 during UV radiation treatment. (A) Spectrum of control *T. distorta* without isotopically labeled tyrosine incubation, (B) spectrum of *T. distorta* after incubation with isotopically labeled tyrosine showing major isotopic mass peaks at $[M+2]^+$ *m/z* 548 and $[M+4]^+$ *m/z* 550, representing the incorporation of one and two tyrosine molecules, respectively and (C) Chemical structure of isotopically labeled L-tyrosine-3,5-d_2 and proposed products derived from incubation with this labeled substrate.

encountered during the large scale culturing conditions. An experiment using L-tryptophan-indole-d_5 (trp-d_5) as the incubated substrate resulted in a modestly enhanced $[M+4]^+$ peak at *m/z* 550 and an intensely enriched $[M+8]^+$ peak at *m/z* 554 (Fig. 4). These enriched peaks represent the incorporation of one or two isotopically labeled tryptophan residues into scytonemin, respectively. The incorporation of only four of the five deuterium atoms present in the tryptophan precursor is expected because cyclization to form the tetracyclic scytonemin monomer is predicted to involve elimination of the hydrogen atom located at C-2 of the indole ring (Fig. 4A). Therefore, enrichment of scytonemin by four or eight mass units from incubation with trp-d_5 is fully consistent with tryptophan incorporation during the biosynthesis of this metabolite.

Interestingly, enrichment from isotopically labeled tryptophan precursors may suggest a limited endogenous biosynthesis of tryptophan, as indicated by the intensity of the $[M+8]^+$ isotopic peak at *m/z* 554. Because both the monoisotopic peak and the $[M+4]^+$ peak have low intensities compared to the $[M+8]^+$ peak, most of the scytonemin produced during these incubation studies is created using the exogenously provided, isotopically labeled tryptophan.

The isotope enrichment study using tyr-d_2 similarly indicated that isotopically labeled substrate is incorporated to a high level due to the intensities of the $[M+2]^+$ peak at *m/z* 548 and the $[M+4]^+$ peak at *m/z* 550. The prephenate dehydrogenase enzyme associated with the scytonemin biosynthetic gene cluster was previously shown to be involved in the transformation of tyrosine into 4-hydroxyphenylpyruvate, an immediate precursor for scytonemin biosynthesis.[10] The high level of incorporation of isotopically labeled tyrosine into scytonemin, despite reports that tyrosine can function as a feedback inhibitor of prephenate dehydrogenase, suggests that other enzymes not associated with the biosynthetic gene cluster, such as tyrosine transaminase, might be utilized to produce the hydroxyphenylpyruvate substrate from exogenous tyrosine.[13] Overall, these observations suggest that the regulation of scytonemin biosynthesis is complex and involves aspects of aromatic amino acid biosynthesis regulation as well as external environmental signals such as UV-irradiance.

To demonstrate that both tyrosine- and tryptophan-derived precursors were independently involved in scytonemin biosynthesis, L-tryptophan-indole-d_5 and L-tyrosine-3,5-d_2 were administered simultaneously. This resulted in incorporation of both substrates into the same scytonemin molecule. MALDI-TOF showed substantial intensity increases for the $[M+4]^+$ peak at *m/z* 550, the $[M+8]^+$ peak at *m/z* 554, and the $[M+12]^+$ peak at *m/z* 558 (Fig. 5). The $[M+4]^+$ peak corresponds to incorporation of either one trp-d_5 subunit or two tyr-d_2 subunits, the $[M+8]^+$ peak corresponds to incorporation of either two trp-d_5 subunits or one trp-d_5 and two tyr-d_2 subunits, and finally, the $[M+12]^+$ peak can only result from the simultaneous incorporation of two trp-d_5 subunits and two tyr-d_2 subunits. These results support tyrosine and tryptophan as independent substrates for scytonemin biosynthesis, and demonstrate the utility of MALDI-TOF analysis of incubation studies using multiple substrates.

MALDI-TOF mass spectrometry was also utilized to gain insights into the in vivo rate of biosynthesis of scytonemin.[14] Using U-$^{13}C_9$, ^{15}N tyrosine as a substrate for incubation studies in *T. distorta*, we measured the rate of biosynthetic incorporation of tyrosine into scytonemin for 6 days. Small tufts of filaments of *T. distorta* were sampled every 8 h and the molecular ion cluster of scytonemin was analyzed using MALDI-TOF MS. The monoisotopic peak for scytonemin at *m/z* 546 reached detectable levels after 24 h of exposure to UV-A radiation. U-$^{13}C_9$, ^{15}N tyrosine was introduced into these cultures after 48 h of UV exposure to ensure that the concentration of scytonemin had reached a detectable level. Previous studies have shown that scytonemin production in the cyanobacteria *Chroococcidiopsis* sp. and *Diplocolon* sp. increases to significant levels after 48 h.[1,3]

The parent peak $[M+8]^+$ for isotopically labeled scytonemin at *m/z* 554 reached detectable levels at 49 h after exposure to UV radiation, only 1 h after the introduction of the labeled substrate into the culture. The percent intensity of the $[M+8]^+$ peak at *m/z* 554 relative to the percent intensity of the $[M+]^+$ peak at *m/z* 546 was measured throughout the six day incubation. Over the first 36 h of the experiment, a steady and significant increase in the higher mass peak was observed (Fig. 6). However, after this initial period, a slow decline in the intensity of the $[M+8]^+$ peak was seen, suggesting that the cyanobacterium had exhausted the supply of exogenous U-$^{13}C_9$, ^{15}N tyrosine. These results illustrate that the incorporation rate of isotopically labeled precursors can be

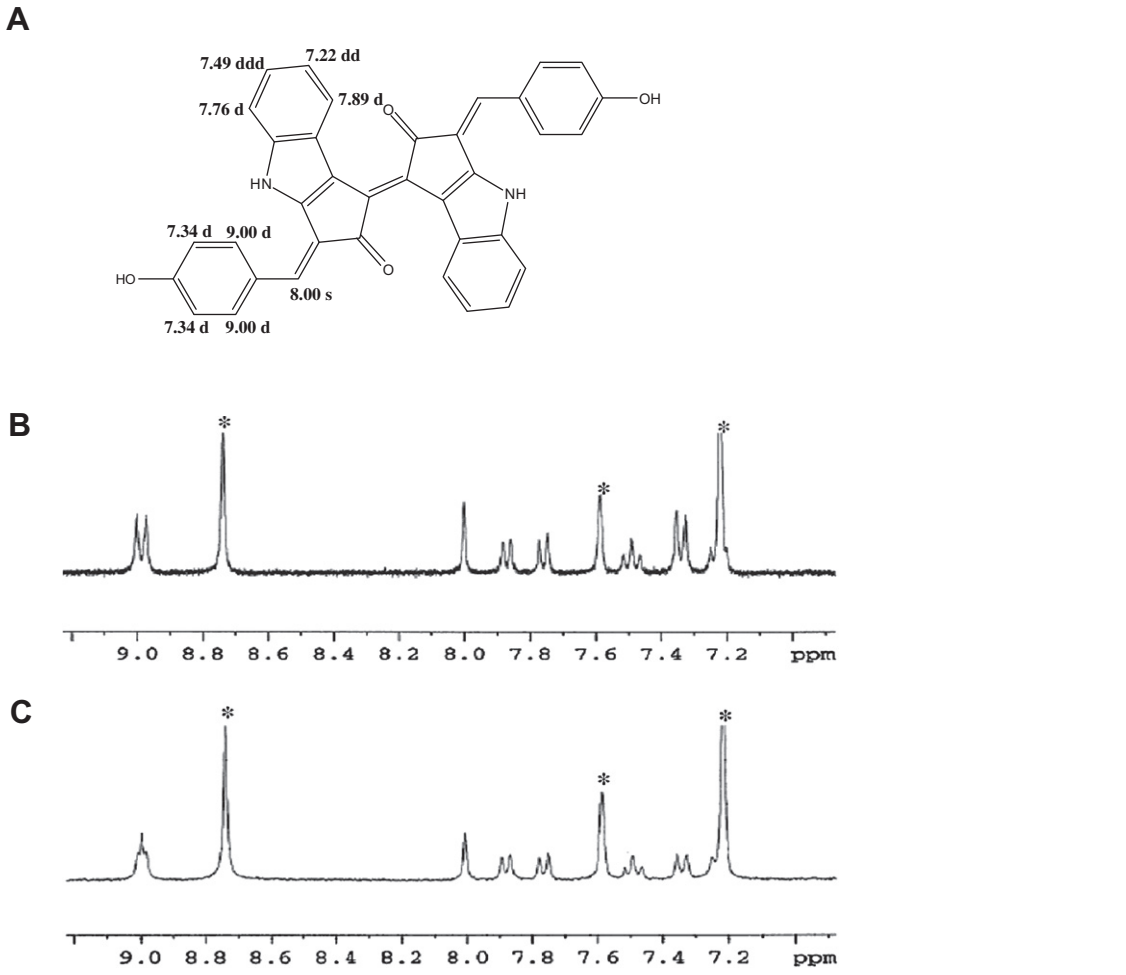

Figure 3. (A) Structure of scytonemin labeled with reported ^{1}H NMR resonances when in pyridine-d_5,[2] (B) ^{1}H NMR of unlabeled scytonemin and (C) ^{1}H NMR of scytonemin after incorporation of L-tyrosine-3,5-d_2. Pyridine solvent peaks are marked with asterisks.

monitored to give insights into the biosynthetic rate and turnover of a secondary metabolite using MALDI-TOF. The ability to monitor in vivo metabolite turnover rates may be useful for understanding how to engineer microorganisms for the production of natural product derived drugs and other renewable resources.

The maximum percent incorporation of isotopically labeled tyrosine into scytonemin was determined to be at 32 h after introduction of the isotopically labeled precursor. Thus, this time point could be used to explore the origin of the ketone carbon formed during condensation of the indolic and phenolic subunits, again employing U-$^{13}C_9$, ^{15}N-tyrosine. Figure 7 shows an $[M+8]^+$ peak at *m/z* 554 and a less intense $[M+16]^+$ peak at *m/z* 562, representing the incorporation of either one or two labeled tyrosine precursors into scytonemin. These results indicate that only 8 of the 10 available isotopically labeled atoms in tyrosine are incorporated into scytonemin. The incorporation of eight tyrosine derived carbons and the previous results showing the loss of the hydrogen at the 2 position of the indole ring in tryptophan confirm that the ketone carbon at position 2 of scytonemin is derived from the tryptophan subunit, and not the tyrosine subunit. These in vivo results support the previous in vitro proposed biosynthetic mechanism where the protein product of Np1276, a gene found in the scytonemin biosynthetic gene cluster, catalyzes the condensation of indole-3-pyruvic acid and hydroxyphenylpyruvic acid through a decarboxylation reaction to form an acyloin. This acyloin product represents the fundamental carbon skeleton required for the formation of the tetracyclic scytonemin monomer.[10,11]

In this study, we showed that both tyrosine and tryptophan are substrates for the biosynthesis of scytonemin, and provide in vivo data to support the decarboxylation of both IPA and HPP, as proposed for the mechanism for the Np1276 gene product.[10] These findings also suggest that scytonemin biosynthesis may involve a complicated regulation of aromatic amino acid biosynthesis and metabolism when exogenous substrates are available. We also identified a new method to analyze isotopically enriched secondary metabolites in cyanobacteria by MALDI-TOF-MS. Only very small quantities of cultured biomass or expensive isotopically labeled substrates were needed in this approach. Moreover, the method was rapid and gave unequivocal results. This technique can also be used to monitor the incorporation of isotopically enriched substrates into secondary metabolites over time, thus giving insight into rates of metabolite production and turnover. MALDI-TOF mass spectrometry coupled with isotope enrichment studies is thus a powerful tool, which can provide keen insights into in vivo biosynthetic processes.

3. Experimental section

3.1. Cyanobacterial strains and culture techniques

The cyanobacteria *N. punctiforme* ATCC 29133 and *Nostoc* sp. ATCC 27893 (PCC7120) were obtained from the American Type Culture Collection (ATCC). The following cyanobacteria were obtained from Carolina Biological Supply: *Gloeocapsa* sp., *Lyngbya* sp. *O. tenius*, *Microcystis aeruginosa*, *Cylindrospermum*

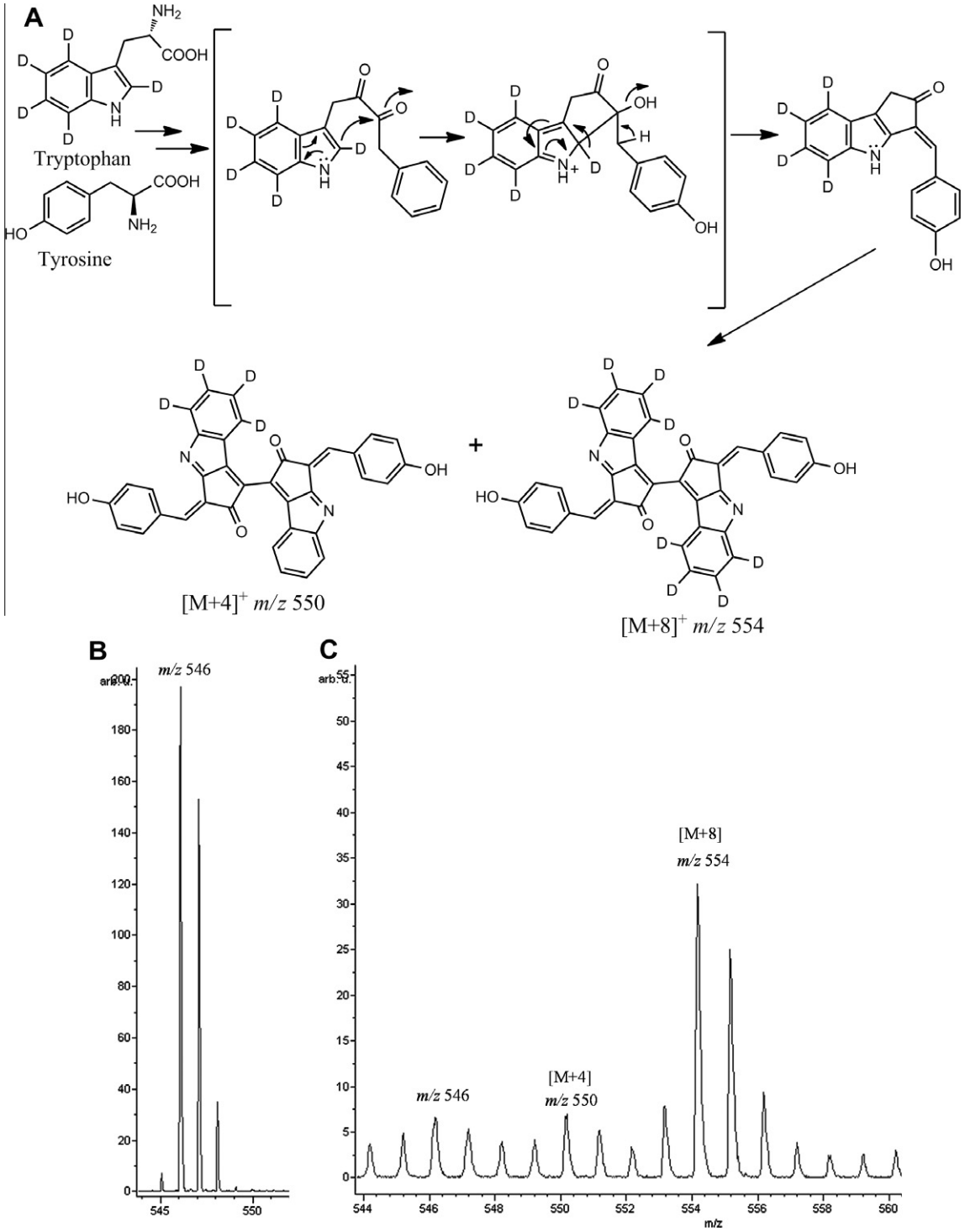

Figure 4. MALDI-TOF spectra of *T. distorta* extract after incubation with L-tryptophan-indole-d_5 during UV stimulation. (A) Scheme showing proposed incorporation of isotopically labeled L-tryptophan-indole-d_5 to give the observed isotopic labeling pattern. The reaction sequence supports the loss of one deuterium atom during the proposed cyclization to form the tetracyclic scytonemin monomer, (B) Control spectrum of *T. distorta* showing the scytonemin monoisotopic peak at *m/z* 546; (C) Spectrum of *T. distorta* after incubation with L-tryptophan-indole-d_5 showing a small amount of scytonemin incorporating one isotopically enriched tryptophan (*m/z* 550) and a significant amount of scytonemin incorporating two isotopically enriched tryptophan subunits (*m/z* 554).

sp., *F. musicola*, *Gloeotrichia* sp., *Anabaena inequalis*, and *T. distorta*. The cultures were maintained in a unialgal condition in liquid BG-11 freshwater media at 20 °C or 29 °C under a light intensity of approximately 19 μmol m^{-2} s^{-1} and a light/dark cycle of 16 h/8 h. Scytonemin production was induced by exposure to 0.64 mW/cm^2 UV-A radiation (λ_{max} = 365 nm) at 29 °C.

3.2. General experimental

Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) spectra were obtained on a Bruker Microflex MALDI-TOF mass spectrometer using approximately 1 μL of MALDI matrix solution (Per 1 mL: 35 mg α-cyano-4-hydroxycinnamic acid

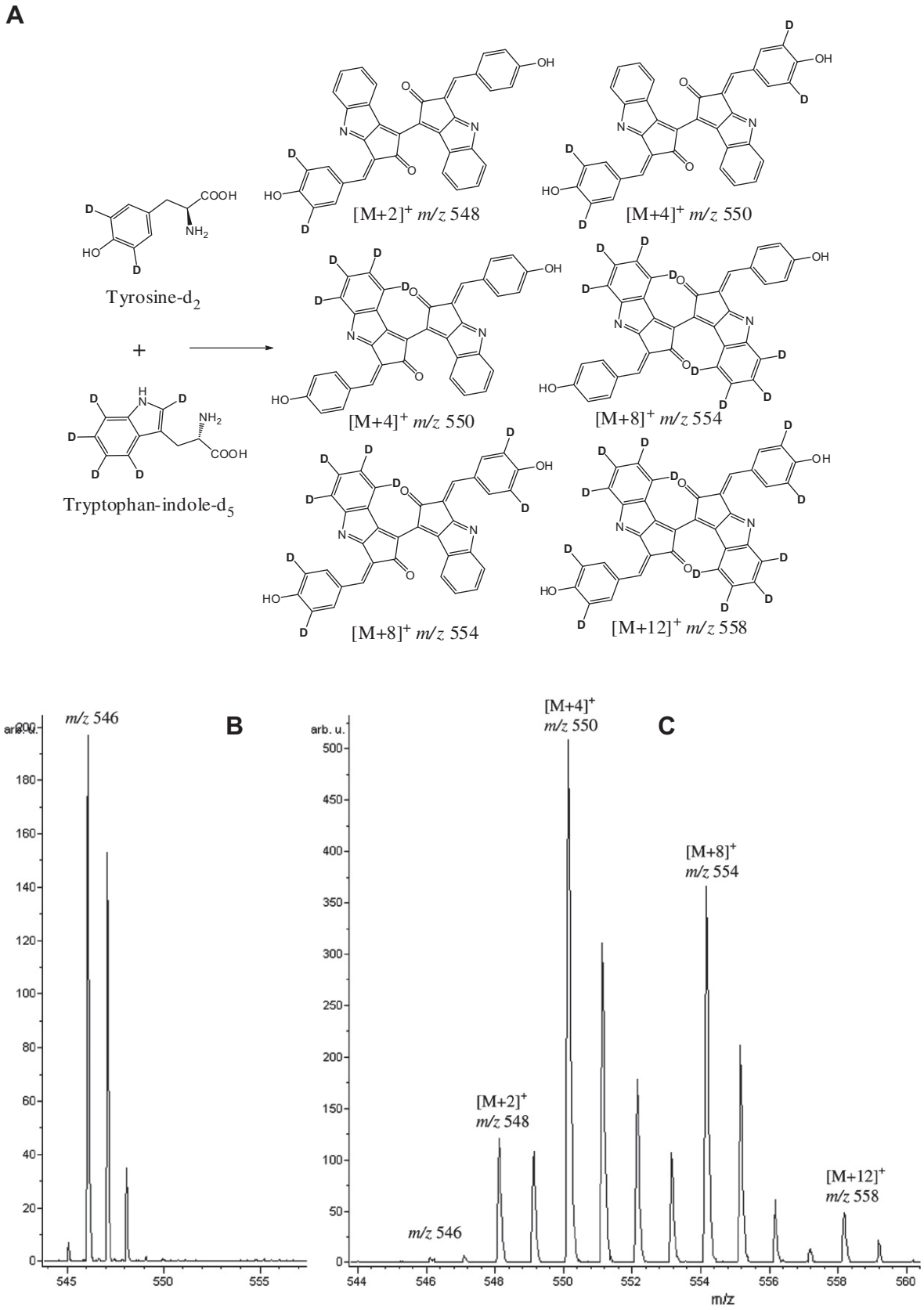

Figure 5. MALDI-TOF spectra of *T. distorta* extract after incubation with L-tryptophan-indole-d_5 and L-tyrosine-d_2 during stimulation by UV radiation. (A) Chemical structures of L-tryptophan-indole-d_5 and L-tyrosine-d_2 showing the proposed products of the major isotopic peaks shown in C, (B) control spectrum of *T. distorta* without incubation with isotopically labeled substrates showing the scytonemin monoisotopic peak at *m/z* 546 and (C) Mass spectrum of *T. distorta* after incubation with isotopically labeled substrates showing significant isotopic peaks at $[M+2]^+$ representing the incorporation of a single tyrosine residue, $[M+4]^+$ representing the incorporation of two tyrosine subunits or a single tryptophan subunit, $[M+8]^+$ representing the incorporation of two tyrosine molecules and one tryptophan subunit or two tryptophan subunits, and $[M+12]^+$ representing the incorporation of two tyrosine and two tryptophan derived subunits.

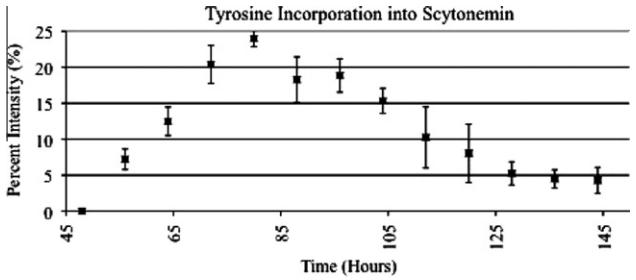

Figure 6. Graphical representation of the percent intensity of the parent peak of labeled scytonemin (*m/z* 554) after incubation with [U-$^{13}C_9$, ^{15}N]tyrosine compared to the monoisotopic peak (*m/z* 546). An increase in the percent intensity between 48 and 84 h represents a high utilization of the labeled substrate for scytonemin biosynthesis. The decline in percent intensity between 84 and 144 h likely represents a decrease in the available isotopically labeled substrate, thus resulting in a decrease in the overall percent of isotopically labeled scytonemin. Standard error is reported from 2 biological samples, one with 3 technical replicates.

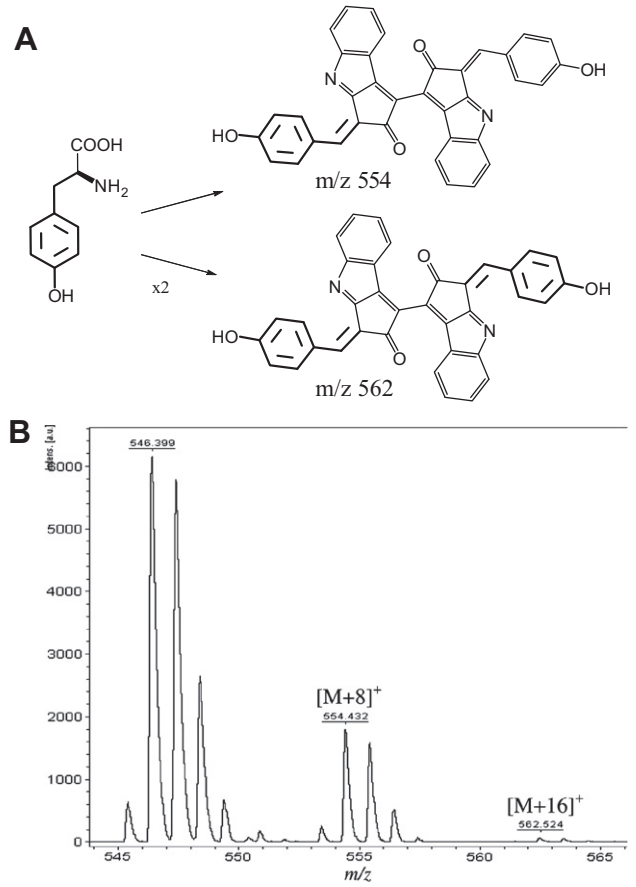

Figure 7. MALDI-TOF mass spectrum showing the incorporation of [U-$^{13}C_9$, ^{15}N]tyrosine into scytonemin in *T. distorta*; (A) Chemical structures of [U-$^{13}C_9$, ^{15}N]tyrosine and the proposed isotopically labeled scytonemin products and (B) mass spectrum of the isotopically labeled scytonemin showing important mass peaks at $[M+8]^+$ representing the incorporation of eight ^{13}C atoms and $[M+16]^+$ representing the incorporation of 16 ^{13}C atoms into the scytonemin chemical skeleton.

(CHCA), 35 mg 2,5-dihydroxybenzoic acid (DHB) (Universal MALDI matrix, Sigma–Aldrich), 750 μL acetonitrile, 248 μL milliQ H_20, 2 μL TFA) per 0.1 μg of biomass. One microliter of this crude matrix solution was deposited on a well (spot) of the Bruker Microflex MSP 96 Stainless Steel Target Plate. After each spot had dried at room temperature, the plate was analyzed using a Bruker Microflex MALDI-TOF mass spectrometer equipped with flexControl 3.0. MALDI-TOF data was analyzed using Clinprotools software (Bruker). Isotopically enriched substrates were purchased from Cambridge Isotope Laboratories: L-tyrosine (ring 3,5-d_2, 98%); L-tyrosine (U-$^{13}C_9$, 97–99%, ^{15}N, 97–99%); L-tryptophan (indole-D_5, 98%).

3.3. L-Tyrosine-3,5-d_2 stable isotope feeding experiment

T. distorta grown for approximately 50 days was exposed to UV-A radiation for 3 days. Tyr-d_2 (3 mg) was added to approximately 10 mL of culture at 48 h, and harvested at 72 h (494.1 mg wet wt). A tuft of *T. distorta* was prepared for MALDI-TOF analysis by dissolving in the MALDI matrix. The remainder was extracted repeatedly with methanol (MeOH) followed by extraction with ethyl acetate (EtOAc) to yield 0.6 mg crude labeled scytonemin. Scytonemin was confirmed by identifying NMR peaks consistent with those reported in the literature.

N. punctiforme ATCC 29133 was grown for approximately 75 days prior to exposure to UV-A radiation for 6 days. L-Tyrosine-3,5-d_2 (Tyr-d_2; 500 mg) was added to approximately 3 L of culture on days 2 and 4, and harvested on day 6 (10.91 g wet wt). The harvested mass was extracted repeatedly with methanol followed by extraction with ethyl acetate to yield 24.3 mg crude scytonemin. The crude scytonemin was repeatedly purified with methanol and hexane to yield 5.4 mg of pure isotopically labeled scytonemin.

3.4. L-Tryptophan-indole-d_5 stable isotope feeding experiment

T. distorta grown for approximately 50 days was exposed to UV-A radiation for 3 days. L-Tryptophan-indole-d_5 (Trp-d_5; 4 mg) was added to approximately 10 mL of culture at 48 h, and harvested at 72 h (415.9 mg wet wt). A tuft of *T. distorta* was prepared for MALDI-TOF analysis by dissolving in MALDI matrix. The remainder was extracted repeatedly with methanol followed by extraction with ethyl acetate to yield 1.7 mg of crude labeled scytonemin.

3.5. Tyrosine-d_2 and tryptophan-indole-d_5 stable isotope feeding experiment

T. distorta grown for approximately 50 days was exposed to UV-A radiation for 3 days. Tyr-d_2 (2 mg) and Trp-d_5 (2 mg) were added to approximately 10 mL of culture at 48 h, and harvested at 72 h (463.8 mg wet wt). A tuft of *T. distorta* was prepared for MALDI-TOF analysis by dissolving in MALDI matrix. The remainder was extracted repeatedly with methanol followed by extraction with ethyl acetate to yield 0.9 mg of crude labeled scytonemin.

3.6. U-$^{13}C_9$, ^{15}N tyrosine stable isotope feeding experiment

T. distorta grown for approximately 65 days was exposed to UV-A radiation for 8 days. U-$^{13}C_9$, ^{15}N-L-Tyrosine (6 mg) was added to approximately 75 mL of culture at 48 h, and harvested at 8 days (3.1 g wet wt). During the first 6 days (144 h), a small tuft of *T. distorta* was removed from the culture every 8 h, flash frozen and stored at −20 °C for future MALDI-TOF analysis. Between 48 h and 64 h, a small tuft of *T. distorta* was removed from the culture every hour, flash frozen and stored at −20 °C for future MALDI-TOF analysis. These samples were prepared for MALDI-TOF analysis by dissolving in MALDI matrix.

MALDI-TOF data were analyzed using Clinprotools software (Bruker) to identify the intensity of the scytonemin mass peak and all isotopic peaks associated with natural abundance of stable isotope incorporation and incorporation of the isotopically labeled substrate. Percent isotopic incorporation of single and double isotopically labeled scytonemin was then calculated using previously established methods.[15] The rate of incorporation of

U-$^{13}C_9$, ^{15}N-tyrosine into scytonemin was determined by averaging the percent intensity of the mass peak at *m/z* 554 based on the monoisotopic peak at *m/z* 546. These data were plotted using Excel (Microsoft).

Acknowledgments

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