Genetically engineered biosynthetic pathways for nonnatural C$_{60}$ carotenoids using C$_5$-elongases and C$_{50}$-cyclases in *Escherichia coli*

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While the majority of the natural carotenoid pigments are based on 40-carbon (C$_{40}$) skeleton, some carotenoids from bacteria have larger C$_{50}$ skeleton, biosynthesized by attaching two isoprene units (C$_5$) to both sides of the C$_{40}$ carotenoid pigment lycopene. Subsequent cyclization reactions result in the production of C$_{50}$ carotenoids with diverse and unique skeletal structures. To produce even larger nonnatural novel carotenoids with C$_{40}$$+$$C_5$$+$$C_5$$=C_{60}$ skeletons, we systematically coexpressed natural C$_{50}$ carotenoid biosynthetic enzymes (lycopene C$_5$-elongases and C$_{50}$-cyclases) from various bacterial sources together with the laboratory-engineered nonnatural C$_{50}$-lycopene pathway in *Escherichia coli*. Among the tested enzymes, the elongases and cyclases from *Micrococcus luteus* exhibited significant activity toward C$_{50}$-lycopene, and yielded the novel carotenoids C$_{60}$-flavuxanthin and C$_{60}$-sarcinaxanthin. Moreover, coexpression of *M. luteus* elongase with *Corynebacterium* cyclase resulted in the production of C$_{60}$-sarcinaxanthin, C$_{60}$-sarprenoxanthin, and C$_{60}$-decaprenoxanthin.

Carotenoids are a class of natural pigments covering yellow, orange and red colors. More than 750 carotenoids have been identified in various plants, fungi, and microorganisms$^1$, and a wide range of essential biological functions have been described, with light-harvesting, photoprotection, antioxidant, and pro-vitamin activities, and roles in the control of membrane fluidity$^2$. In recent years, carotenoids have been increasingly considered in applications as therapeutic or bioelectronic materials$^3$,$^4$. Small changes in carotenoid structures have been associated with large differences in material properties$^6$ and biological activities$^3$,$^6$. Hence, the discovery of the structurally novel carotenoids is eagerly awaited.

Most carotenoids have C$_{40}$ backbones, although a few microbial carotenoids have C$_{40}$ backbones. The structural diversity of carotenoids is mainly derived from differences in desaturation levels of the C$_{40}$ backbone, types of end groups, and functional patterns of cyclization, oxidation, and esterification. Further structural diversity of carotenoids can be achieved by liberating carotenoids from their C$_{40}$ backbones. For example, carotenoid cleavage enzymes can be used to produce a range of carotenoids with different-backbone sizes (C$_{14}$, C$_{20}$, or C$_{28}$ carbons)$^7$. These include precursors of bixin (C$_{24}$), crocetin (C$_{30}$), retinals (C$_{30}$), and other hormonal compounds such as strigolactones (C$_{15}$) and abscisic acid (C$_{15}$), as reviewed previously$^8$.

Another source of structure and size diversity comes from the attachment of additional isoprene units (C$_5$) to C$_{40}$ carotenoids. Since C$_{50}$ and C$_{60}$ carotenoids were first reported in the 1960s$^{8,9}$, many of these carotenoids have been identified in prokaryotes, including gram-positive bacteria such as *Micrococcus*,$^{10}$ *Corynebacterium*,$^{10}$ *Dietzia*,$^{12}$ and extreme halophilic archaea such as *Halobacterium* or *Haloarcula*.$^{13}$ Although the natural functions of these carotenoids are not fully understood, contributions to cell membrane fluidity and permeability have been suggested$^{13}$. In addition, the attachment of C$_5$ units reportedly confers remarkable antioxidant activities to carotenoids$^{14}$, and the resulting compounds have been considered for their potential as therapeutic agents$^{13-15}$.
To date, four natural biosynthetic pathways for C50 carotenoids have been reported. All known C50 carotenoids are derived from lycopene, a four-step enzymatic desaturation product of phytoene, which is produced by the condensation of two molecules of geranylgeranyl diphosphate (C20). One pathway from extremely halophilic archaea *Haloarcula japonica* yields acyclic C50 carotenoid bacterioruberin as the final product, whereas three others produce cyclic C50 carotenoids (Fig. 1a). In the *Micrococcus luteus* carotenoid biosynthetic pathway, lycopene elongase (CrtE2) attaches two additional isoprene units (DMAPP) to the 2,2′-position of lycopene to produce the acyclic C50 carotenoid flavuxanthin, and subsequent β-cyclization by cyclase (CrtYeYf) produces sarcinaxanthin (β,β-rings)10. C50 carotenoid synthesis in *Corynebacterium* also proceeds via the flavuxanthin intermediate, but the expressed cyclase (CrtYeYf) is less specific and yields a mixture of decaprenoxanthin (ε,ε-rings), sarprenoxanthin (ε,γ-rings) and sarcinaxanthin, when expressed in *E. coli*. In contrast, the bacterium *Dietzia*, reportedly produces C50 carotenoids with β,β-rings (C.p.450)12 via a different acyclic intermediate (Fig. 1a).

In a previous study, we constructed a nonnatural C50 backbone carotenoid pathway in which two geranyl-farnesyl diphosphates (C20PP) are condensed to produce C50-phytoene (Fig. 1b). This compound is subsequently subjected to a six-step desaturation reaction resulting in the synthesis of C50-lycopene. Using a metabolic filtering approach, we extended this C50-lycopene pathway to specifically produce the nonnatural C50 backbone carotenoids C50-β-carotene, C50-zeaxanthin, C50-canthaxanthin, and C50-astaxanthin. In this study, we systematically expressed elongase and cyclase in engineered *E. coli* strains harboring enzymes of the C50-lycopene synthetic pathway and established pathways for the synthesis of the novel carotenoids C60-flavuxanthin, C60-sarcinaxanthin, C60-sarprenoxanthin, and C60-decaprenoxanthin (Fig. 1b).
Results

Activities of elongase and cyclase in the natural C40 pathway. We selected Corynebacterium glutamicum, M. luteus, and Dietzia sp. CQ4 as sources of lycopene elongases and C50 cyclases, because the C50 carotenoid pathways from these organisms have been previously reconstructed in E. coli10–12. The carotenoid cluster (shown in Supplementary Fig. 1) of C. glutamicum11 comprises crtEb (lycopene elongase), crtYeYf (heterodimeric C50 ε/γ-cyclase), and lycopene-producing genes (crtEBI). The expression of this gene cluster in E. coli produces sarprenoxanthin, decaprenoxanthin, and sarcinaxanthin via the acyclic intermediate flavuxanthin10,11 (Fig. 1a). The corresponding gene cluster in M. luteus10 encodes crtE2 (lycopene elongase) and crtYgYh (C50 γ-cyclase), and coexpression of these genes with crtEBI results in specific accumulation of sarcinaxanthin10. Uniquely, the carotenogenic gene cluster of Dietzia sp. CQ412 encodes a gene for elongase (lbtC) that is fused in frame with a single subunit of cyclase (lbtB), which forms a heterodimer with another unit of cyclase (lbtA). Expression of lbtB with an lbtA gene product results in the production of a functional C50 β-cyclase12.

Although different in organization, all of the elongase and cyclase genes are clustered in small <1.7-kb regions (Supplementary Fig. 1), allowing one-step PCR amplification from genomic DNA. We cloned these DNA fragments into a plasmid that encodes Pantoea ananatis phytoene desaturase variant CRTIN304P, a variant that can desaturate both (C40-) phytoene and C50-phytoene (see Fig. 2a for plasmid construct). In addition to these three operons (for C. glutamicum, M. luteus and Dietzia sp.), we cloned the elongase and cyclase genes from Corynebacterium efficiens. The carotenoid gene cluster of this organism has exactly the same organization as that of C. glutamicum, and in our homology analyses, DNA sequence identity of the two clusters (from crtE to crtEb, see Supplementary Fig. 1) was 64.3%. Yet, the functions of the C. efficiens carotenoid pathway and its products remain uncharacterized.

To characterize the functions of elongase and cyclase in the context of natural (C40) pathway (Fig. 1a) in E. coli, we used an engineered E. coli strain that constitutively expresses fdsY81M and crtMF26A, W38A genes (crtEB equivalent17) and produces phytoene (C40). We introduced plasmids encoding phytoene desaturase (a crtI variant), elongases and cyclases into this strain, and after culturing for 48 h, we extracted carotenoids using acetone and
analyzed these using high performance liquid chromatography (HPLC) (Fig. 2a). These analyses showed that cells expressing *C. glutamicum* *crt*Ye*Yf*Eb produced a mixture of (*C*50*) sarcinaxanthin (2), (*C*40*) sarpenoxanthin (3), and (*C*30*) decaprenoxanthin (4), with significant quantities of their substrate (*C*40*) lycopene (1). Cells expressing *C. efficiens* *crt*Ye*Yf*Eb indistinguishable composition of carotenoids as those from the *C. glutamicum* gene cluster. Cells expressing *M. luteus* *crt*E2*Yg*Yh produced a single peak of (*C*40*) sarpenaxanthin (2), whereas expression of *Dietzia* sp. CQ4 *lbt*ABC resulted in the specific production of *C*.p.450 (*C*40*) (Table 1). Despite the differences in the source of lycopene biosynthetic genes, promoters, RBS sequences, *E. coli* strain or culture condition, product distribution of the pathway we constructed was very similar to those from previous reports16. To determine whether these enzymes can metabolize *C*50*-lycopene, we coexpressed plasmids encoding the elongase and cyclase enzymes in another *E. coli* strain that constitutively expresses *fds*V157A, *v1275A* and *crt*M-*FAB,CBA,DAN,F235S* and produces *C*50*-phytoene17. In this strain, the phytoene desaturase mutant (*Crt*IN304P) can desaturate *C*50*-phytoene to produce *C*50*-lycopene selectively17, providing a sole substrate for the elongase and cyclases in this strain. However, we observed no novel peaks but *C*50*-lycopene in carotenoid fractions from this strain.

### Ribosome binding site (RBS)-optimized cyclase/elongase results in higher production of cyclic *C*50*-carotenoids.

In the previous section, we observed (*C*40*) lycopene accumulated in cells expressing elongase and cyclases (Fig. 2a), suggesting the presence of inefficiencies of this pathway, and room for improvement. The present four carotenoid operon constructs share similar operon organization, in which open reading frames (ORF) for cyclases and elongases are partially overlapping. Specifically, the start codon of *M. luteus* *crt*Yg is 28 base pairs upstream of the stop codon of the *crt*E2 gene, and the start codon of *crt*Yh overlaps with the stop codon of the *crt*Yg gene. In *C. glutamicum* and *C. efficiens*, *crt*Ye and *crt*Yf overlap by 4 bases, and in *Dietzia*, *lbt*ABC genes also overlap by 4 bases, as is commonly observed in various gene clusters containing carotenoid operons18. We investigated translation initiation rates using the RBS calculator18–20 and found RBS scores as low as 0.01 (Table 1), suggesting very low translation initiation rates and likely formation of stable secondary mRNA structures. In the native operon with overlaps in translational stop and start of ORFs, the translational re-initiation is likely ensuring efficient translation *in vivo*.

To improve expression levels of elongases and cyclases in *E. coli*, we redesigned our artificial operons (Fig. 2b) by (1) separating the ORFs to yield tandem and distinct non-overlapping reading frames, and (2) to provide stronger RBSs, designed using RBS calculator to have RBS strengths between 1,000 - 5,000 (Table 1). These RBS strengths range were chosen since it was previously confirmed optimal for expressing various carotenoid biosynthetic genes in the plasmid backbone we use in this study16–20 (i.e. p15A, pUC). When expressed with (*C*40*) lycopene pathways, all of these new constructs with engineered RBSs led to improved production of natural *C*50*carotenoids and significant reductions in unconverted lycopene contents (Fig. 2b).

### Production of nonnatural cyclic *C*60*carotenoids.

Following the RBS engineering of elongases and cyclases, we cotransformed the new constructs into the strains that selectively synthesize *C*50*-phytoene (Fig. 3). From the cells expressing *M. luteus* *crt*E2*Yg*Yh, we observed novel peaks 7 and 9 in HPLC chromatograms, and these were eluted earlier than that of *C*50*-lycopene (6) (Fig. 3a,b). Peak 9 exhibited absorbance maxima at 496 nm; shorter than that for *C*50*-lycopene (9, 512 nm). The *m/z* value (837) of peak 9 matched that of *C*50*-sarcinaxanthin (*C*50*H*84*O*2, *C*50*H*85*O*2 calcd. for 837.6550) (Supplementary Fig. 2). The structure of this carotenoid was determined as *C*50*-sarcinaxanthin using nuclear magnetic resonance (1H-NMR) with correlation spectroscopy (COSY) and rotating frame overhause effect spectroscopy (ROESY) (Table 2). In addition, we identified peak 7 as the *C*60 pathway intermediate *C*55*-nonflavuvaxanthin based on an absorbance maximum of 513 nm and a *m/z* value of 753 (*C*53*H*39*O*).

| Organism     | Gene       | RBS strength in v1 plasmid | Designed RBS strength in v2 plasmid |
|--------------|------------|----------------------------|-------------------------------------|
| *M. luteus*  | *crt*E2    | 812                        | 4296                                |
|              | *crt*Yg    | 0.01                       | 1525                                |
|              | *crt*Yh    | 140                        | 4700                                |
| *Dietzia* sp. CQ4 | *lbt*A    | 968                        | 4106                                |
|              | *lbt*BC   | 15                         | 4700                                |
| *C. glutamicum* | *crt*Ye   | 4493                       | 3891                                |
|              | *crt*Yf   | 4342                       | 5886                                |
|              | *crt*Eb   | 389                        | 5066                                |
| *C. efficiens* | *crt*Ye   | 776                        | 1113                                |
|              | *crt*Yf   | 33                         | 2663                                |
|              | *crt*Eb   | 93                         | 1990                                |

Table 1. Ribosome binding site strengths of original and designed operons. RBS strength obtained using a ribosome binding site (RBS) calculator18–20. The RBS and open reading frame (ORF) sequences are listed in Supplementary Tables S1 and S2.
The crtE2 gene expression (without crtYgYh) from *M. luteus* resulted in a new peak 8 with an m/z value of 838 and an absorbance maximum of 513 nm (Fig. 3g). The carotenoid from peak 8 showed absorption maxima at 460, 494, and 527 nm (Fig. 3h). Positive ion ESI TOF MS at m/z 857.6107 \( \text{M}^+ \) revealed the molecular formula \( \text{C}_{60}\text{H}_{82}\text{O}_2 \) (\( \text{C}_{60}\text{H}_{82}\text{O}_2\text{Na} \) calcd. for 857.6212) (Supplementary Fig. 3) for this carotenoid, and structural analyses using 1H-NMR with COSY and ROESY revealed the carotenoid C60-flavuxanthin (Table 2).

We did not observe any novel carotenoid peaks in experiments using elongase and cyclase genes from *C. glutamicum*, *C. efficiens*, or *Dietzia* sp. CQ4 (Fig. 3c–e). Hence, the lycopene elongases (CrtEb in *Corynebacterium* and LbtC in *Dietzia* sp. CQ4) at least fail to convert C50-lycopene in *E. coli*. However, the activities of the cyclases (CrtYeYf from *Corynebacterium* and LbtAB from *Dietzia* sp. CQ4) were still unknown, since the first elongase step failed to provide the substrate (C60-flavuxanthin or C60-C.p.496) for these cyclase enzymes.

Because *Corynebacterium* and *M. luteus* pathways share the intermediate flavuxanthin, we generated a chimeric operon containing *M. luteus* crtE2 and *C. efficiens* crtYeYf. Following expression in cells, we detected three carotenoid peaks (Fig. 3f) (9, 10, and 11) with identical absorption spectra (Fig. 3h), indicating the presence of the same chromophore. Although we could not determine the NMR spectroscopy of the compound from the associated chromatographic peaks, their absorption spectra, m/z values (838) and retention times strongly indicate that peaks 9, 10, and 11 were C60-sarcinaxanthin (as shown in Fig. 3b), C60-sarprenoxanthin, and C60-decaprenoxanthin, respectively. These results also indicate that cyclases (crtYeYf) from *Corynebacterium* are functional in the C50-to-C60 pathway.

**Discussion**

In this study, we demonstrated that carotenoid elongation and cyclization enzymes from natural C50 carotenoid pathways metabolize C50-lycopene to novel nonnatural C60 carotenoids. These carotenoids (C60-flavuxanthin, C60-sarcinaxanthin, C60-sarprenoxanthin, and C60-decaprenoxanthin) are larger than any known natural carotenoids, and their absorbance spectra are red-shifted by as much as 58 nm compared with the natural counterpart (440 nm vs. 496 nm). With rare \( \gamma \)-ring structures, these C60 carotenoids provide a unique set of accessible carotenoid structures with as yet unknown functions. Natural C60 carotenoids were previously discovered in thicker membranes of halophilic bacteria, which survive in extreme hypersaline and low-temperature environments\(^{13,14,16}\). The present nonnatural C60 carotenoids are interesting candidates for functional characterization in extremophiles. These future studies may lend understanding to the roles of long-chain carotenoids in nature.

Through the coexpression of natural carotenoid enzymes, we have increased the number of laboratory-generated C50 carotenoid pathways. Many carotenoid enzymes from C50 and C30 pathways exhibit...
Table 2. $^1$H NMR data for $C_{60}$-sarcinaxanthin and $C_{60}$-flavuxanthin in CDCl$_3$. See Supplementary Fig. S4 for the numbering of carotenoid structure.

| Position | $C_{60}$-Sarcinaxanthin (9) | | Position | $C_{60}$-Flavuxanthin (8) |
|----------|-------------------------------|------------------|--------------------------|------------------|
| H-2 (2') | 1.28 m                        | H-2 (2')         | 2.08 m                   |
| H-3 (3') | 1.18 m                        | H$_2$-3 (3')     | 1.56 m                   |
| H-4 (4') | 1.71 m                        | H$_2$-4 (4')     | 2.00 m                   |
|          | 2.05 m                        |                  |                          |
| H-6 (6') | 2.48 d                        | 10 H-6 (6')      | 5.93 d                   |
| H-7 (7') | 5.83 d                        | 15,5,10 H-7 (7')| 6.48 dd                 |
| H-8 (8') | 6.12 d                        | 15.5 H-8 (8')   | 6.24 d                   |
| H-10 (10') | 6.12 d                      | 12 H-10 (10')   | 6.18 d                   |
| H-11 (11') | 6.62 dd                     | 15,12 H-11 (11')| 6.63 dd                 |
| H-12 (12') | 6.34 d                       | 15 H-12 (12')  | 6.36 d                   |
| H-14 (14') | 6.23 d                      | 11 H-14 (14')  | 6.23 d                   |
| H-15 (15') | 6.64 dd                     | 15,11 H-15 (15')| 6.64 dd                |
| H-16 (16') | 6.38 d                      | 15 H-16 (16')  | 6.38 d                   |
| H-18 (18') | 6.27 br. d               | 10 H-18 (18')  | 6.27 br. d              |
| H-19 (19') | 6.64 m                       | H-19 (19')      | 6.64 m                   |
| H$_2$-20 (20') | 0.96 s                  | H-20 (20')     | 4.70 br. S              |
|          |                               |                 | 4.78 br. S              |
| H$_2$-21 (21') | 0.73 s                 | H$_2$-21 (21') | 1.63 s                  |
| H-22 (22') | 4.53 s                       | H$_2$-22 (22') | 1.80 s                  |
| H-22 (22') | 4.76 s                       |                  |                         |
| H$_2$-23 (23') | 1.98/1.99 s         | H$_2$-23 (23') | 1.97 s                  |
| H$_2$-24 (24') | 1.98/1.99 s         | H$_2$-24 (24') | 1.98/1.99 s             |
| H$_2$-25 (25') | 1.98/1.97 s         | H$_2$-25 (25') | 1.98/1.97 s             |
| H-26 (26') | 1.72 m                       |                  |                         |
| H-26 (26') | 2.24 dd                     | 14,5,5 H-26 (26')| 2.10 m                |
| H-27 (27') | 5.43 m                       | H-27 (27')      | 5.36 s                   |
| H$_2$-29 (29') | 1.67 s                   | H$_2$-29 (29') | 1.67 s                  |
| H$_2$-30 (30') | 4.03 s                   | H$_2$-30 (30') | 4.00 s                  |

significant substrate promiscuity and accept a wide range of substrates with recognizable locally specific structures. Consequently, these enzymes are active in nonnatural pathway contexts without any mutations. However, there is increasing reports on carotenoid modifying enzymes that exhibit unexpected selectivity against non-cognate but very similar substrates. For example, lycopene cyclases from plants (LCYe) cyclize only one end of the acyclic substrate lycopene and leave the other end un-cyclized. Hence, ε-cyclases likely possess mechanisms for avoiding cyclization of non-cognate substrates. Similarly, the β-carotene 15,15′ cleavage enzyme BCMO1 only accepts β-carotene (β,β-end) as a substrate and does not act on ε-carotene (ε,ε-end). However, after removing the ε-end via 9′,10′ cleavage by BCMO2, BCMO1 precisely cleaves the 15,15′ bond and liberates retinal.

While the elongases from *Corynebacterium* and *Dietzia* sp. CQ4 exhibited a significant activity towards natural substrate (C$_{40}$-lycopene), they did not show any activity towards C$_{50}$-lycopene. Considering that enzymes from both strains have significant activity in the C$_{60}$-to-C$_{50}$ context, it is unlikely that their inability to act on C$_{50}$-lycopene reflects a lack of activity in *E. coli*. The more likely alternative is that some of these enzymes have higher substrate/size specificity and resist C$_{50}$-lycopene as a substrate. Kim et al. showed that the *Corynebacterium* elongase CrtEb is functional in another non-cognate context and acts on the ψ-end of the C$_{50}$ carotenoid 4,4′-diaponeurosporene. Given this unpredictability of promiscuous functions toward non-cognate substrates, studies of several accessible gene candidates are required to identify genes that perform intended non-natural tasks. Also, it is interesting how or whether *Dietzia* elongase and cyclase and *Corynebacterium* elongase, which were found non-functional in C$_{50}$-to-C$_{60}$ context in the present work, can acquire these new activities by mutations.

Methods

**Strains and reagents.** *E. coli* XL10-Gold cells were used for cloning, and XL1-Blue cells were used for carotenoid production. All enzymes were purchased from New England Biolabs. Lennox-LB Broth Base was purchased from Life Technologies, Bacto™ Yeast Extract, and Bacto™ Tryptone were purchased from BD Biosciences, and all other chemicals and reagents were obtained from Nacalai Tesque (Kyoto, Japan). The antibiotics carbenicillin and chloramphenicol were used at 30 and 50 µg/mL, respectively.
Plasmid construction. pUCara-crtIN304P, a plasmid encoding a crtIN304P gene downstream of an arabionose inducible araBAD promoter, was derived from a previous study. The plasmids pUCara-crtIN304P-crtEYeYEdEc, pUCara-crtIN304P-crtE2YgYEd, and pUCara-crtIN304P-lbtABC-CQ4 were constructed by amplifying genes for elongase and cyclase from various sources using the primers listed in Supplementary Table 1 and cloning them into the ApaI/SpeI restriction site of pUCara-crtIN304P. The plasmids pAC-fdsHVaYVa-crtIN304P and pAC-fdsHvM-crtIN304P, derived from the previous study, were then inserted into the ApaI/SpeI restriction site of pUCara-crtIN304P without spacer sequences.

Culture conditions. Single colonies were inoculated into 2 ml of LB media with antibiotics in culture tubes and were shaken at 37°C for 16 h. Overnight cultures of 2 ml were diluted 100-fold into 40 ml of fresh Terrific Broth media in 200 ml flasks and were then shaken at 200 rpm in an incubator at 30°C. After 8 h, 0.2% (w/v) arabinoinducer was added and cells were cultured for an additional 40 h.

Product extraction and purification. Cell cultures were centrifuged at 3,270 × g for 15 min at 4°C. Cell pellets were washed with 10 ml of 0.9% (w/v) NaClq and were then repelleted by centrifugation. Products were extracted by vigorously vortexing for 5 min in 10 ml of acetone containing 30-mg/L butylated hydroxytoluene. One-ml aliquots of hexane and 35-ml aliquots of 1% (w/v) NaClaq were then added, and the samples were centrifuged at 3,250 × g for 15 min. After collecting the product-containing hexane phase, the solvent was evaporated in a vacuum concentrator. Extracts were finally dissolved in 15–50-μl aliquots of tetrahydrofuran:methanol (6:4) for further analysis.

HPLC–MS analysis for compound identification and quantification. Aliquots (2–15 μL) of final extracts were analyzed using Shimadzu Prominence HPLC system equipped with LCMS-2020 MS spectrometer, a photodiode array (PDA) detector with Waters Spherisorb ODS2 Analytical Columns (4.6 × 250 mm, 5 μm, PSS831915). Mobile phases comprised acetonitrile/tetrahydrofuran/methanol (58:7:35) at a flow rate of 2 mL/min for further analysis.

Data Availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**

S.W., L.L., M.F. and D.U. conceived and designed the study. S.W. and L.L. performed experiments. M.F., L.L. and D.U. analyzed the results. T.M. performed NMR and MS/MS analysis. S.K.N., K.S. and D.U. supervised the study. M.F., L.L. and D.U. wrote the paper.

**Additional Information**

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