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Studies on the biosynthesis of paraherquamide. Construction of the amino acid framework

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Abstract—It has been previously established in this laboratory that the β-methyl-β-hydroxyproline moiety of the potent anthelmintic agent paraherquamide A, is biosynthetically derived from L-isoleucine. The downstream events from L-Ile to paraherquamide A have now been investigated. The synthesis of [1-^{13}C]-labeled L-β-methylproline is described by means of a Hoffman–Loeffler–Freytag reaction sequence from [1-^{13}C]-L-Ile. This amino acid is shown to be a direct biosynthetic precursor to paraherquamide A by feeding and incorporation experiments in growing cultures of *Penicillium fellutanum*. Three tryptophan-containing dipeptides of L-β-methylproline have been constructed: [$^{13}C_2$]-2-(1,1-dimethyl-2-propenyl)-L-tryptophanyl-3(*S*)-methyl-L-proline; [$^{13}C_2$]-3(*S*)-methyl-L-prolyl-2-(1,1-dimethyl-2-propenyl)-L-tryptophan and [$^{13}C_2$]-*cyclo*-2-(1,1-dimethyl-2-propenyl)-L-tryptophan-3(*S*)-methyl-L-proline. [α-^{15}N, 1-^{13}C]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophan was also prepared but none of these substances were found to serve as biosynthetic precursors to paraherquamide A.

1. Introduction

The paraherquamides are a group of heptacyclic mycotoxins isolated from various *Penicillium* sp. that possess potent anti-parasitic activity[1,2]. Members of the paraherquamide family include paraherquamide A (**1**), which displays the most potent anthelmintic activity of these compounds, paraherquamides B-G (**2**–**4**, **7**, **10**, **11**), VM55595 (**12**), VM55596 (**5**), VM55597 (**6**), VM55599 (**13**), SB203105 (**8**), and SB200437 (**9**). Although similar in structure, the paraherquamides vary with respect to oxygenation and substitution of both the proline ring and the prenylated oxindole ring. As part of an ongoing investigation into the biosynthesis of this family of alkaloids, the construction of the amino acid framework leading to the bicyclo[2.2.2]ring system of paraherquamide A has been investigated and the results of this study are disclosed here (Fig. 1).[3,4]

To provide some perspective and background concerning the biogenesis of the paraherquamides, the biosynthesis of a simpler, structurally related alkaloid, brevianamide A (**17**), has been the subject of previous study in our laboratory.[5] In 1974, Birch and coworkers found that [5-^{3}H]-L-proline (**14**), [methylene-^{3}H]-D,L-tryptophan (**15**) and *cyclo*-[*methylene*-^{14}C]L-tryptophanyl-[5-^{3}H]-L-proline (**16**) were biosynthetically incorporated into brevianamide A (**17**) in significant radiochemical yield (Scheme 1).[6]

Later, Williams and co-workers showed that [8-$^{3}H_2$]deoxybrevianamide E (**18**) was also incorporated into brevianamide A (**17**).[5b,c] Based on the structural similarities of these substances, we initially speculated that L-tryptophan and L-proline might also provide the biosynthetic building blocks of paraherquamide A with L-tryptophan providing the oxindole moiety and L-proline forming the framework of the β-methyl-β-hydroxyproline moiety. The hypothesis that L-proline could serve as the precursor to the β-methylproline ring could be based on studies conducted by Arigoni and co-workers, who studied the biosynthesis of the β-methylproline residue in the cyclic peptide antibiotic bottromycin (**19**), a natural substance isolated from *Streptomyces bottropenis* (Fig. 2). These studies demonstrated that [*methyl*-^{13}C]-L-methionine (**20**) was incorporated into bottromycin giving rise to the 3(*R*)-methyl group of the β-methyl proline ring of bottromycin with a specific incorporation of 20–25%.[7] In principle, methylation of the β-position of proline in paraherquamide biosynthesis might arise via methyl transfer from S-adenosylmethionine to a 2,3-dehydroproline derivative followed by reduction. However, the ETH research group has demonstrated that in the instance of bottromycin biosynthesis, the mechanism for methylation involves a B_{12}-type radical coupling mechanism that does not implicate loss of the prolyl α-proton.

Keywords: biosynthesis; secondary metabolism; feeding experiments.
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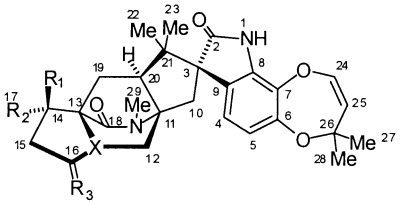
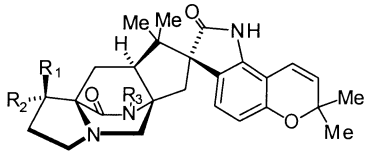
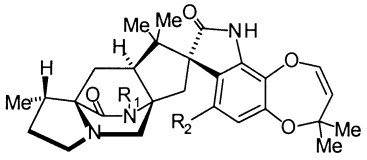


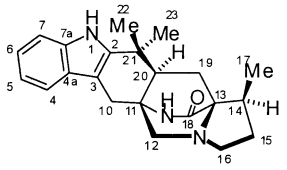
1, (−)-paraherquamide A, R_1 = OH, R_2 = Me, R_3 = H_2, X = N
2, (−)-paraherquamide B, R_1 = H, R_2 = H, R_3 = H_2, X = N
3, (−)-paraherquamide C, R_1 = R_2 = CH_2, R_3 = H_2, X = N
4, (−)-paraherquamide D, R_1 = O, R_2 = CH_2, R_3 = H_2, X = N
5, VM55596, R_1 = OH, R_2 = Me, R_3 = H_2, X = N^+-O^-
6, VM55597, R_1 = OH, R_2 = Me, R_3 = O, X = N



10, paraherquamide F, R_1 = H, R_2 = Me, R_3 = Me (VM55594)
11, paraherquamide G, R_1 = OH, R_2 = Me, R_3 = Me (VM54158)
12, VM55595, R_1 = H, R_2 = Me, R_3 = H



7,VM54159, R_1 = Me, R_2 = H (paraherquamide E)
8, SB203105, R_1 = Me, R_2 = OH
9, SB200437, R_1 = H, R_2 = H

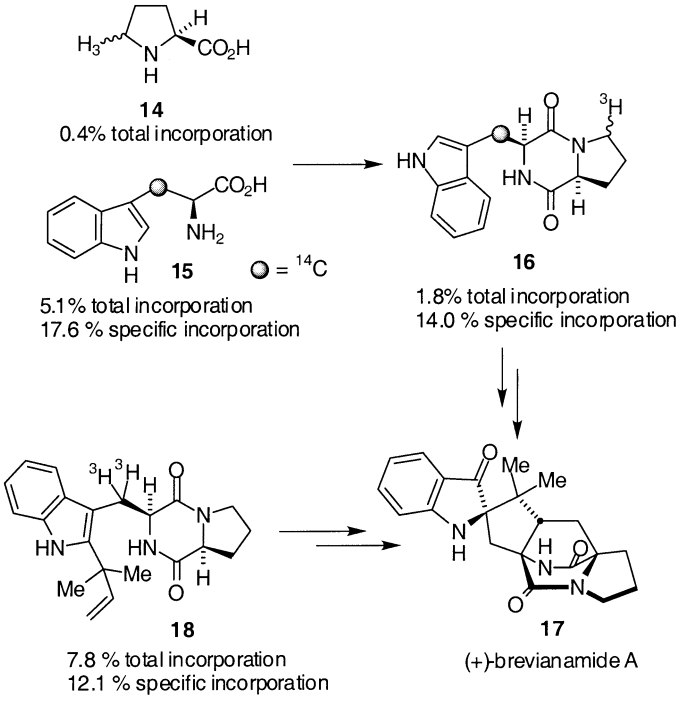


13, VM55599

Figure 1.

We previously reported that S-adenosylmethionine only contributes the *N*-methyl group (C-29) in the paraherquamide A structure and does not donate the C-17 methyl group. This led us to speculate that the β-methyl proline moiety of paraherquamide may instead be derived from L-isoleucine through an oxidative cyclization process.[3b] Both possible progenitors of the β-methyl-β-hydroxyproline ring, L-proline and L-isoleucine, were explored through ^{13}C labeling studies and only L-isoleucine was found to provide the β-methyl-β-hydroxyproline portion of paraherquamide A.

In addition to the amino acid constituents, paraherquamide A also contains two five carbon isoprene-derived units: one comprising the dioxepin ring system and one forming the unusual bicyclo [2.2.2] diazaoctane ring system. We have proposed that the latter isoprene group is attached to the 2-position of the indole via an 'inverse' prenyl transferase



Scheme 1.

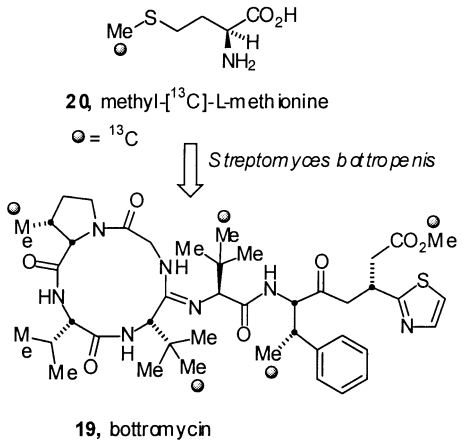


Figure 2.

the temporal sequence of events in building up the complex polycyclic array of the paraherquamide family.

2. Results and discussion

To determine what the primary amino acid building blocks of paraherquamide A are, feeding experiments were performed on *Penicillium fellutanum* (ATCC: 20841) with [1-^{13}C]-L-tryptophan (**21**), [*methyl*-^{13}C]-L-methionine (**22**), [1-^{13}C]-L-proline (**23**) and [1-^{13}C]-L-isoleucine (**24**) (Fig. 3, Table 1). The position and the percentage of ^{13}C incorporation in paraherquamide A was determined by means of ^{13}C NMR spectroscopy.[†] The [1-^{13}C]-L-tryptophan (**21**) was incorporated, into the oxindole ring as expected, (2.5% incorporation) with the label at C-12. The [*methyl*-^{13}C]-L-methionine (**22**) was not incorporated in the β-methyl-proline ring, but rather, only at C-29, the *N*-methyl position

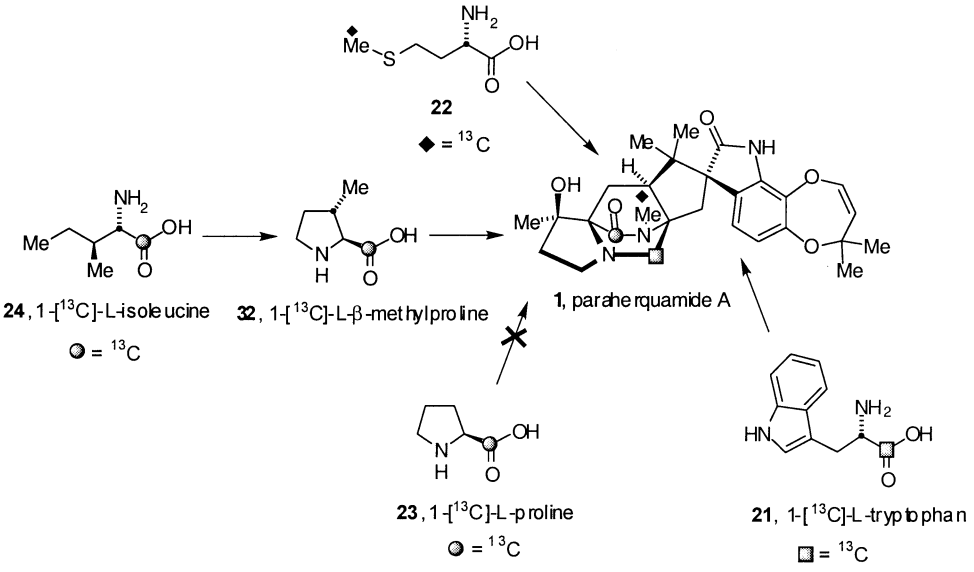


Figure 3.

Table 1. Results for the feeding experiments with compounds **21**–**27** and **32**

Compound	mmol	**1** produced (mmol)	Incorporation at C-18 (%)	Incorporation at C-12 (%)	Incorporation at C-29 (%)
21	0.152	0.041	–	2.5	–
22	0.164	0.049	–	–	0.66
23	0.149	0.011	0	–	–
24	0.155	0.053	3.7	–	–
24	0.157	0. 028	3.3	–	–
25	0.081	0.019	1.8	0.4	–
25	0.028	0.023	1.3	0.8	–
26	0.120	0.017	1.2	0.5	–
26	0.091	0.014	1.7	0.9	–
27	0.043	0.042	0	0	–
32	0.169	0.041	14.6	–	–

that subsequently undergoes a [4+2] cycloaddition across an azadiene system derived from the amino acids to form the bicyclo [2.2.2] ring system.[8] Because of the importance of this isoprene unit in forming the unusual core structure of paraherquamide A, several different potential metabolites were explored containing the 'inverse' prenyl group at the 2-position of the indole which might give an indication of

of the monoketopiperazine ring (0.6%) and [1-^{13}C]-L-proline (**23**) did not show any incorporation. On the other hand, feeding of [1-^{13}C]-L-isoleucine (**24**) to *Penicillium*

[†] The percentage of incorporation was also determined through electrospray mass spectrometry. See Section 4 for the incorporation levels and method of determination.

fellutanum followed by harvesting the cells and isolation of paraherquamide A, revealed that the labeled L-isoleucine was incorporated to the extent of 3.3–3.7% with the label at C-18. It was thus clear from the feeding experiments with the primary amino acids that L-isoleucine and not L-proline serves as the precursor to the β-methyl-β-hydroxyproline ring. However, it still remained unclear at what point of the biosynthetic pathway L-isoleucine was converted into β-methylproline.

We therefore set out to determine if the oxidative cyclization of the α-amino group of L-Ile on the side chain ethyl residue occurs before or after condensation with L-tryptophan. The doubly labeled dipeptides [$^{13}C_2$]-L-isoleucyl-L-tryptophan (**25**), [$^{13}C_2$]-L-tryptophanyl-L-isoleucine (**26**), and [2,5-$^{13}C_2$]-cyclo-L-tryptophan-L-isoleucine (**27**) were synthesized and fed to *P. fellutanum* to test the latter possibility.[3b] As a result, no incorporation was detected by means of ^{13}C NMR for the diketopiperazine **27**. However, ^{13}C NMR data showed incorporation for the dipeptides **25** and **26**, namely 1.2–1.8% incorporation at C-18 and 0.4–0.9% incorporation at C-12, respectively. The ^{13}C NMR spectra of the paraherquamide A isolated from these feeding experiments did not provide compelling evidence for site-specific incorporation of both labels from the intact dipeptides. The observed levels of incorporation are more consistent with hydrolysis of the dipeptides and re-incorporation of the individual amino acids presumably coupled with additional metabolic degradation and reconstitution of ^{13}C-enriched building blocks. Furthermore, incorporation levels determined from the mass spectra of Paraherquamide A corroborated the findings that the doubly labeled metabolites were not incorporated intact (see Table 3 in Section 4 for results) (Fig. 4).

Since none of the dipeptides were incorporated intact, it therefore seemed logical that oxidative cyclization of L-isoleucine must *precede* coupling to L-tryptophan.[9] Synthesis of [1-^{13}C]-3(*S*)-methyl-L-proline (**32**) from [1-^{13}C]-L-isoleucine (**24**) was accomplished according to the method of Lavergne et al. (Scheme 2).[10] Treatment of [1-^{13}C]-L-isoleucine ethyl ester (**28**) with *t*-butylhypochlorite

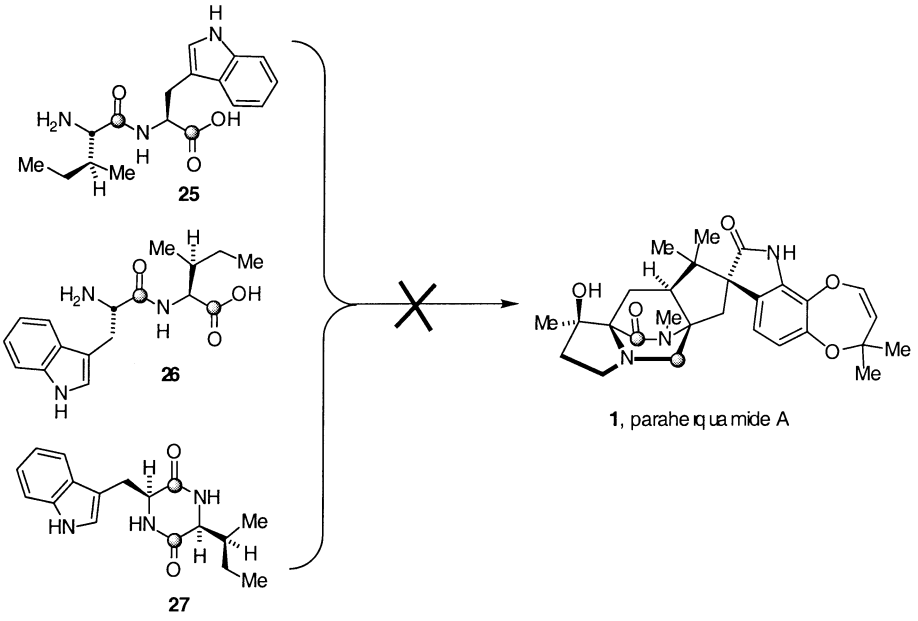
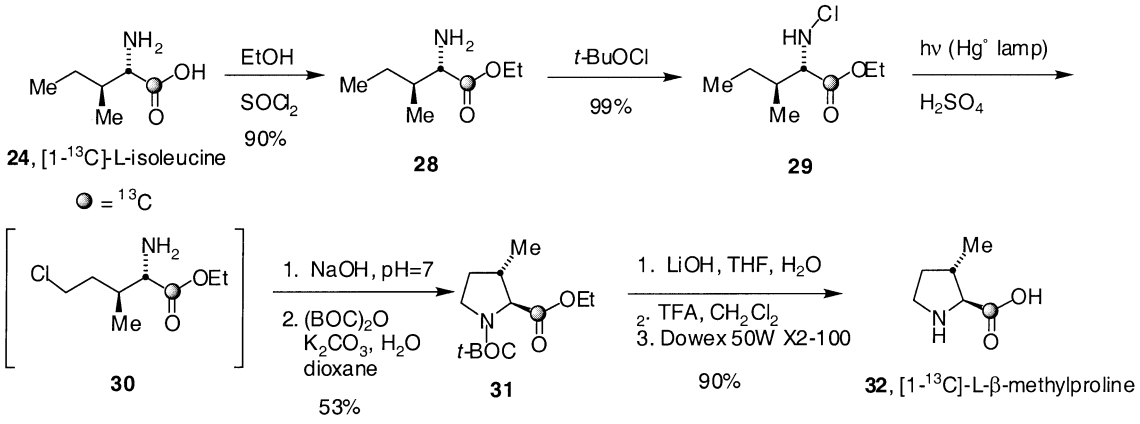


Figure 4.



Scheme 2.

afforded the N-chlorinated derivative (**29**) which, after exposure to a mercury lamp in 85% H_2SO_4, gave the Hoffman–Loffler–Freytag intermediate (**30**). Upon neutralization, this intermediate suffered spontaneous cyclization to give the desired labeled β-methyl-proline ethyl ester. The reaction mixture was treated with di-*tert*-butyldicarbonate to facilitate separation of the desired product from any unreacted [1-^{13}C]-L-isoleucine ethyl ester. Hydrolysis of the purified ethyl ester **31** with LiOH in aqueous THF followed by removal of the *t*-Boc with TFA and ion exchange chromatography (Dowex 50WX2-100) afforded the desired labeled [1-^{13}C]-3(*S*)-methyl-L-proline (**32**) as the free amino acid.

A feeding experiment on *P. fellutanum* with [1-^{13}C]-3(*S*)-methyl-L-proline (**32**) gave paraherquamide A with 14.6% incorporation of the labeled precursor. A significantly higher level of incorporation was observed in this case, when compared with [1-^{13}C]-L-isoleucine (**24**) (14.6 vs. 3.7%, respectively). This is consistent with the postulate that 3(*S*)-methyl-L-proline is biosynthesized from L-isoleucine and is therefore farther along the pathway to paraherquamide A. In addition, 3(*S*)-methyl-L-proline is a non-proteinogenic amino acid that is most likely used exclusively in the production of paraherquamide A and related compounds, whereas L-isoleucine must be consumed in the biosynthesis of proteins and other primary and/or secondary metabolites in the fungus. Thus, **32** serves as a more efficient precursor to paraherquamide.

Once it was determined that L-isoleucine is first converted to β-methylproline before coupling to L-tryptophan, more advanced intermediates on the biosynthetic pathway were considered. We have recently demonstrated that the addition of the first equivalent of dimethylallyl pyrophosphate (DMAPP) occurs in formation of the bicyclo [2.2.2] ring system and that the C_5 unit that forms part of the dioxepin system is installed later.[3c] There are numerous stages at which the addition of the inverted isoprene unit to the indole system might occur; several of these possibilities are outlined in Fig. 5. Inverted prenylation of the indole nucleus might occur after formation of the diketopiperazine **35** in analogy to what has been established experimentally for the structurally related brevianamides, to form **36**. Alternatively, either L-tryptophan itself or the dipeptides L-tryptophanyl-3(*S*)-methyl-L-proline or 3(*S*)-methyl-L-prolyl-L-tryptophan might be prenylated, to give rise to potential intermediates **37**–**39**. To explore these possibilities, the doubly isotopically labeled compounds **35**–**39** were synthesized and fed to cultures of *P. fellutanum*.

The diketopiperazine **35** was synthesized as outlined in

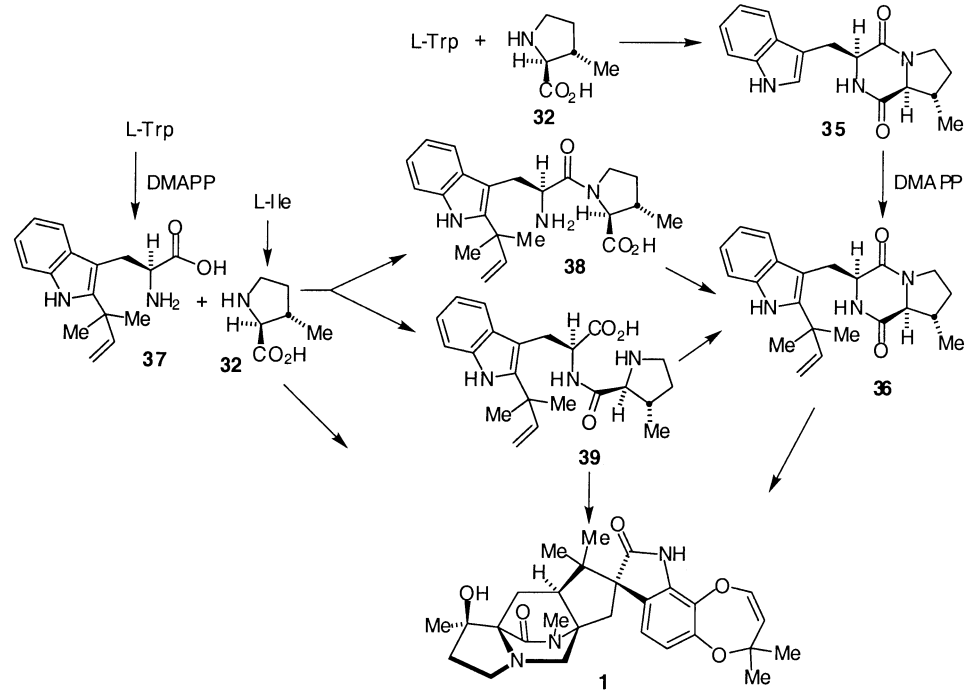
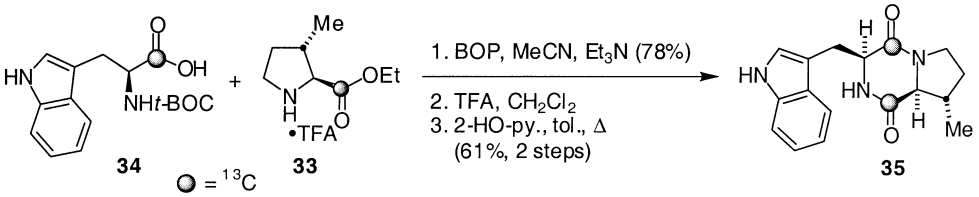
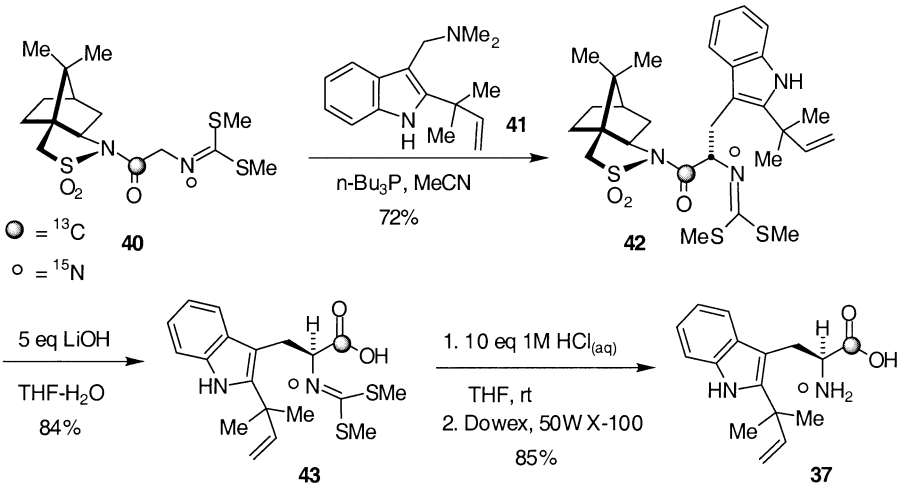


Figure 5.



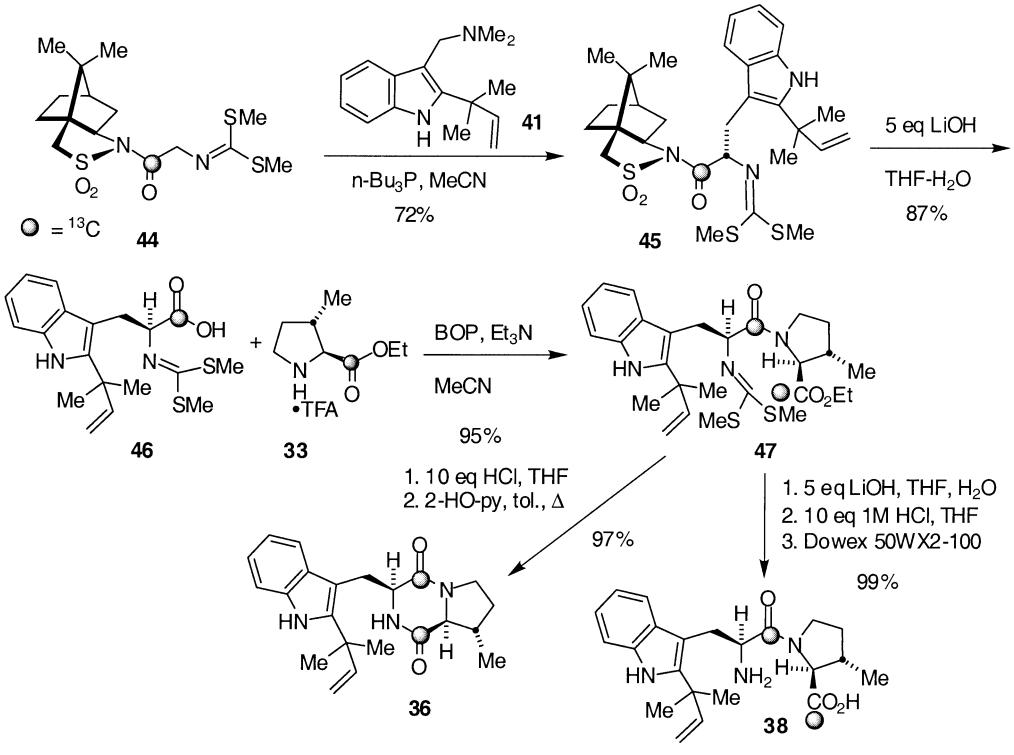
Scheme 3.



Scheme 4.

Scheme 3. Coupling of the *N*-*t*-Boc protected [1-^{13}C]-L-tryptophan (**34**) to [1-^{13}C]-3(*S*)-methyl-L-proline ethyl ester (**33**) was accomplished in good yield with the BOP reagent. Removal of the *t*-Boc group with TFA and cyclization in refluxing toluene containing a catalytic amount of 2-hydroxypyridine afforded **35** in 48% yield over the three steps.

Labeled 2-(1,1-dimethyl-2-propenyl)-L-tryptophan (**37**) was synthesized from the labeled Oppolzer sultam glycinate as shown in Scheme 4.[11] This facilitated the preparation of either the singly labeled ^{13}C-tryptophan derivative or the doubly labeled ^{13}C, ^{15}N-derivative. In the case of 2-(1,1-dimethyl-2-propenyl)-L-tryptophan itself, the doubly ^{13}C, ^{15}N-labeled material was chosen to explore the question whether or not the tryptophanyl moiety was oxidatively deaminated at some point in the biosynthesis (specifically in the formation of the azadiene system for the proposed intramolecular Diels–Alder cyclization reaction). The retention or loss of the ^{15}N-label could therefore provide significant mechanistic insight into the coupling and cyclization with 3(*S*)-methyl-L-proline. The synthesis of **37** was accomplished via a Somei-type coupling[12] reaction of **40** with **41** and 0.3 equiv. of PBu_3 in refluxing acetonitrile over a period of 8 h (Scheme 4). The sultam moiety of **42** was removed through hydrolysis in a 2:1 mixture of THF and water with 5 equiv. of LiOH for 24 h. Purification of **43** was accomplished by flash column chromatography which,



Scheme 5.

in turn, allowed recovery of the chiral sultam. The *N*-dithiomethylmethylene group was removed with 10 equiv. of 1N HCl in THF at room temperature over 24 h. The solvent was removed in vacuo and the HCl salt was submitted to Dowex ion exchange chromatography to provide the free amino acid **37**.

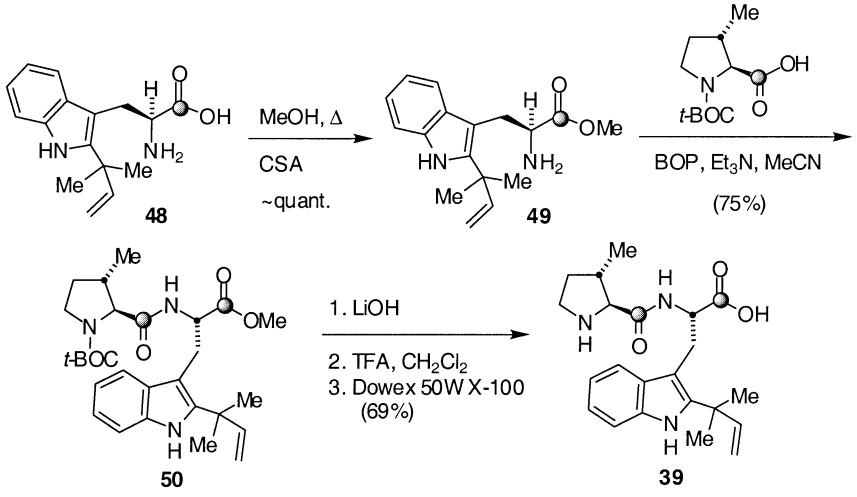
The doubly labeled diketopiperazine of 2-(1,1-dimethyl-2-propenyl)-L-tryptophan and 3(*S*)-methyl-L-proline was prepared according to the procedure outlined in Scheme 5. The intermediate **45** was prepared in the same manner as **42** in 86% yield. After removal of the sultam with LiOH, the BOP reagent was used to carry out its coupling to **33** in 95% yield. Removal of the *N*-dithiomethylmethylene protecting group followed by cyclization in refluxing toluene with a catalytic amount of 2-hydroxypyridine afforded the diketopiperazine (**36**) in excellent yield. Alternatively, the dipeptide L-tryptophanyl-3(*S*)-methyl-L-proline (**38**) was prepared in essentially quantitative yield through deprotection of **47** with LiOH, followed by 1 M HCl and Dowex ion exchange chromatography.

The dipeptide [$^{13}C_2$]-3(*S*)-methyl-L-prolyl-L-tryptophan (**39**) was synthesized according to the procedure outlined in Scheme 6. [1-^{13}C]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophan (**48**) was prepared in the same manner as **37** in 77% yield. Protection of the carboxyl group of **48** as the methyl ester, followed by coupling to [1-^{13}C]-*N*-Boc-3(*S*)-methyl-L-proline with the BOP reagent provided the dipeptide **50**. Deprotection of the methyl ester with LiOH followed by deprotection of the Boc group with TFA and subsequent ion exchange with Dowex provided the dipeptide **39** in 69% yield.

Feeding experiments were performed with the potential intermediates **35**–**39** (see Table 2). Somewhat surprisingly, *none* of these potential precursors were incorporated intact. However, ^{13}C NMR data indicated that while the dipeptides **38** and **39** showed incorporation at C-18 in 0.44 and 0.92%, respectively, no incorporation at C-12 was observed. This again suggests that the dipeptides are catabolized and 3(*S*)-methyl-L-proline is re-incorporated, but the reverse prenylated L-tryptophan is not. The lack of incorporation does not rigorously exclude the intermediacy of **35**–**39** in the biosynthesis of paraherquamide A since penetration of the cell envelope may severely limit uptake of these candidate precursors. On the other hand, dipeptides **38** and **39**, presumably release the reverse prenylated tryptophan derivative **48** inside the cell upon catabolic proteolysis and the lack of incorporation of this moiety either intact from the dipeptides or as an individual amino acid would cast doubt on this species as being a biosynthetic building block.

3. Conclusions

Through feeding experiments with *P. fellutanum*, the primary amino acid building blocks of paraherquamide A have been determined: L-tryptophan, L-methionine and L-isoleucine. L-Isoleucine appears to undergo an oxidative cyclization process to be converted into β-methylproline, which is then efficiently incorporated into paraherquamide A. The hydroxylation of the β-position of the β-methylproline moiety must therefore occur with net retention of stereochemistry. However, we have recently shown that the doubly ^{13}C-labeled cycloadduct **51** (Fig. 6) is incorporated



Scheme 6.

Table 2. Results for the feeding experiments with compounds **35**–**39**

Compound	mmol	1 produced (mmol)	Incorporation at C-18 (%)	Incorporation at C-12 (%)
35	0.042	0.028	0	0
36	0.059	0.035	0	0
37	0.098	0.026	0	–
38	0.040	0.010	0.44	0
39	0.031	0.015	0.92	0

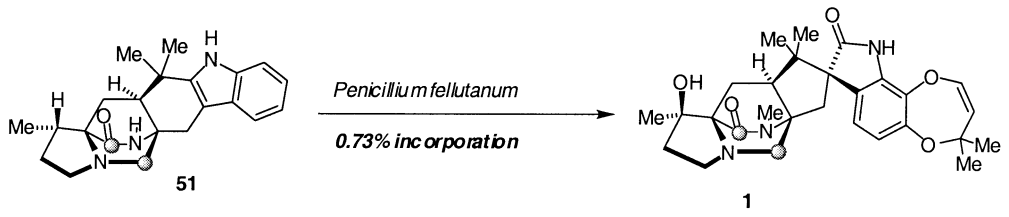
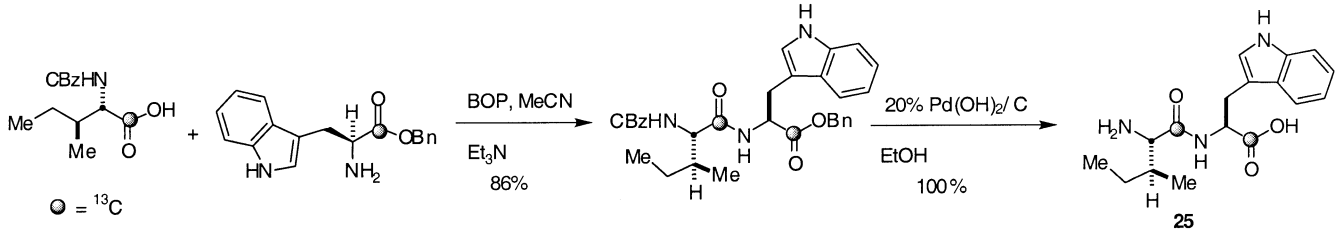


Figure 6.



Scheme 7.

intact into paraherquamide A in *P. fellutanum*,[3c] which would dictate that the hydroxylation of β-methylproline must occur *after* formation of the bicyclo [2.2.2] diazaoctane ring system.

The free amino acid [1-^{13}C]-L-tryptophan was incorporated and thus constitutes the precursor for the oxindole ring. Furthermore, [*methyl*-^{13}C]-L-methionine incorporated exclusively at the *N*-methyl position (C29). The results of feeding experiments with labeled [$^{13}C_2$]-L-isoleucyl-L-tryptophan [$^{13}C_2$]-L-tryptophanyl-L-isoleucine, [2,5-$^{13}C_2$]-*cyclo*-L-tryptophan-L-isoleucine and [1-^{13}C]-β-methyl-L-proline suggest that L-isoleucine is first converted to β-methyl-L-proline before coupling to a tryptophanyl derivative in the biosynthesis of paraherquamide A. Since none of the potential reverse prenylated candidates (**35**–**39**) were incorporated intact, the timing of the addition of the isoprene moiety that culminates in the formation of the bicyclo [2.2.2] ring system remains unclear. It is thus not unreasonable to expect that a multi-domain non-ribosomal peptide synthetase, that would neither charge nor release the putative dipeptide intermediates, is involved in the early stages of paraherquamide biosynthesis leading up to **51**.[13] Studies aimed at clarifying the intermediate metabolites in paraherquamide biosynthesis preceding the formation of metabolite **51** are currently under investigation in these laboratories.

4. Experimental

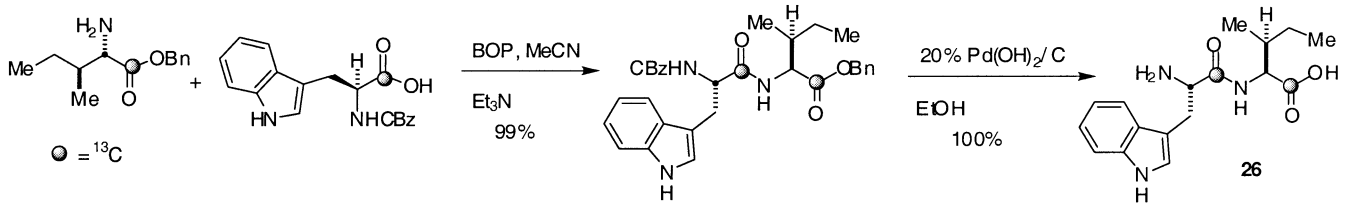
4.1. General considerations

[1-^{13}C]-L-Tryptophan, [*methyl*-^{13}C]-L-methionine and [1-^{13}C]-L-proline were obtained from the Los Alamos NIH Stable Isotopes Resource. [1-^{13}C]-L-isoleucine, 90% enrichment, was obtained from the Los Alamos NIH Stable Isotopes Resource; [1-^{13}C]-L-isoleucine, 99% enrichment, was purchased from Cambridge Isotope Labs. All other reagents were commercial grade and used without purification unless otherwise noted. BOP reagent (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) was purchased from Aldrich Chemical Co. ^{1}H- and ^{13}C NMR spectra were obtained on a Bruker AC300P 300 MHz NMR spectrometer, a Mercury 300 or Inova 400 Varian NMR spectrometer at Colorado State University. NMR spectra were taken in $CDCl_3$ (^{1}H, 7.24 ppm; ^{13}C, 77.0 ppm), CD_3OD (^{1}H, 4.87 ppm, ^{13}C, 49.15 ppm), and d_6-DMSO (^{1}H, 2.50 ppm, ^{13}C, 39.51 ppm) obtained from Cambridge Isotope Labs. Electrospray mass spectra were obtained with a FisonsVG AutoSpec spectrometer. Exact masses were obtained with a VG Quattro-SQ spectrometer. IR spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrometer. EM Science Silica Gel 60 was used for column chromatography and PTLC were performed with EM Science precoated TLC plates (Kiselgel 60, F_{254}, 0.25 mm).

4.2. Synthesis of [$^{13}C_2$]-L-isoleucyl-L-tryptophan

Prepared according to the route described in Scheme 7.

4.2.1. [1-^{13}C]-*N*-Benzyloxycarbonyl-L-isoleucine. [1-^{13}C]-L-Isoleucine (32.1 mg, 0.244 mmol) was dissolved in dd H_2O (75 μL) and 5 M NaOH (50 μL). The reaction mixture was cooled to 0°C while stirring. Benzyl chloroformate (38.5 μL, 0.270 mmol) and 2 M NaOH (135 μL) were added dropwise simultaneously under an argon atmosphere. After addition was complete, the reaction was brought to room temperature slowly and stirred for an additional 2 h. The reaction mixture was then adjusted to pH 10 with saturated aqueous Na_2CO_3 and extracted four times with diethyl ether. The aqueous layer was acidified with 2 M HCl to pH 3 and extracted into diethyl ether four times. The ethereal layer was dried over anhydrous magnesium sulfate, filtered, and the solvent removed in vacuo to provide a colorless oil. Yield: 57.0 mg, 88%. ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.91 (3H, t, J=7.3 Hz), 0.92 (3H, d, J=6.6 Hz), 1.27 (1H, m), 1.49 (1H, m), 1.84 (1H, m), 4.00 (1H, dd, J=6.9, 6.9 Hz), 5.08 (2H, s), 6.68 (1 H, bs), 7.36 (5H, s), 11.1 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.8, 15.7, 25.1, 38.0, 58.5 (d, J_{C-C}=55 Hz), 67.8, 128.3, 128.5, 128.6, 136.3, 156.5, 177.3. IR (NaCl, CH_2Cl_2): 3313, 3047, 2966,



Scheme 8.

1714, 1520, 1455, 1415, 1339, 1092, 739, 697 cm^{-1} $[\alpha]_D^{25}=+6°$ (c=0.0125, CH_2Cl_2).‡

4.2.2. [1-^{13}C]-L-Tryptophan benzyl ester. [1-^{13}C]-L-Tryptophan (44 mg, 0.214 mmol), *p*-toluenesulfonic acid monohydrate (102 mg, 0.536 mmol), benzyl alcohol (250 μL), and dry toluene (15 mL) were refluxed under an argon atmosphere in a Dean–Stark apparatus half filled with activated 4 Å molecular sieves for 12 h. The toluene was evaporated under reduced pressure and the residue was covered with a layer of EtOAc. Saturated aqueous sodium carbonate was then added to make the solution basic. The aqueous layer was separated from the organic layer and extracted three more times with EtOAc. After drying with anhydrous sodium sulfate, the solvent was removed in vacuo. Yield: 34 mg, 54%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a cream colored solid, mp 74–75°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.71 (2H, bs, D_2O exch.), 3.08 (1H, dd, J=7.3, 14.4 Hz), 3.27 (1H, dd, J=5, 14.4 Hz), 3.87 (1H, bs), 5.12 (2H, s), 6.91 (1 H, d, J=2 Hz), 7.10 (1H, ddd, J=1, 7, 7 Hz), 7.18 (1H, ddd, J=1, 7, 7 Hz), 7.25 (1H, d J=6 Hz), 7.26, (1H, m), 7.32 (2H, d, J=6 Hz), 7.32 (2H, t, J=4 Hz), 7.60 (1H, d, J=7 Hz), 8.21 (1H, bs, D_2O exch.). ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.0, 55.4 (d, J_{C-C}=60 Hz),, 66.9, 77.4, 111.3, 111.4, 119.0, 122.4, 123.1, 127.8, 128.5, 128.5, 128.8, 135.9, 136.5, 175.6. IR (NaCl, CH_2Cl_2): 3389, 3161, 3047, 1731, 1586, 1451, 1177, 742, 695 cm^{-1}. $[\alpha]_D^{25}=+30.8°$ (c=0.0065, CH_2Cl_2).[2]

4.2.3. [$^{13}C_2$]-*N*-Benzyloxycarbonyl-L-isoleucyl-L-tryptophan benzyl ester. [1-^{13}C]-L-Tryptophan benzyl ester (34.0 mg, 0.115 mmol) was added to [1-^{13}C]-L-*N*-benzyloxycarbonyl-isoleucine (35.0 mg, 0.131 mmol) dissolved in acetonitrile (5 mL). Triethylamine (24 μL, 0.172 mmol) and BOP reagent (50.92 mg, 0.115 mmol) were added to the flask. After stirring for 2 h at room temperature, brine (10 mL) was added to the reaction mixture. The solution was extracted three times with EtOAc. The combined EtOAc layers were extracted with 2 M HCl, water, 0.5 M $NaHCO_3$, and brine successively. The EtOAc fraction was dried over $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by means of radial chromatography with a 3% mixture of methanol in CH_2Cl_2. Yield: 51.8 mg, 86%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a white solid, mp 152–153°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81 (3H, t J=7 Hz), 0.86 (3H, d, J=7 Hz), 1.04 (1H, m), 1.27 (1H, m), 1.79 (1H, m), 3.27 (1H, dd, J=5, 15 Hz), 3.34 (1H, dd, J=5, 15 Hz), 4.04 (1 H, dd, J=6, 10 Hz), 4.98 (1H, ddd, J=6, 6, 8 Hz), 5.08 (4H, s), 5.35 (1H, d, J=9 Hz, D_2O exch.), 6.43 (1H, d, J=8 Hz, D_2O exch.), 6.75 (1H, bs), 7.08 (1H, t, J=8 Hz), 7.18 (1H, ddd, J=1, 8, 8 Hz), 7.29 (11H, m), 7.50 (1H, d, J=8 Hz), 7.96 (1H, bs, D_2O exch.). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 15.5, 24.9, 27.9, 37.9, 53.1 (d, J_{C-C}=61 Hz), 59.8 (d, J_{C-C}=52 Hz), 67.2, 67.5, 109.7, 111.5, 118.7, 120.0, 122.5, 123.4, 127.7, 128.3, 128.4, 128.7, 128.7, 128.8, 135.4, 136.3, 156.4, 171.1, 171.6. IR (NaCl, CH_2Cl_2): 3319, 3045, 2946, 1710, 1655, 1523, 1452, 1348, 1227, 1101, 1029, 738, 694 cm^{-1}. $[\alpha]_D^{25}=+12.7°$ (c=0.01, CH_2Cl_2).

4.2.4. [$^{13}C_2$]-L-Isoleucyl-L-tryptophan. [$^{13}C_2$]-Z-L-Isoleucine-L-tryptophan benzyl ester (15.4 mg, 0.028 mmol) was suspended with 20% $Pd(OH)_2$/C (10 mg) in 2 mL of 95% ethanol. After bubbling first argon and then $H_{2(g)}$ through the solution, it was placed under $H_{2(g)}$ atmosphere with an $H_{2(g)}$ balloon. The reaction was determined to be complete by means of TLC after 16 h. The reaction mixture was filtered through a pad of Celite and evaporated to dryness. Yield: 9 mg, 100%. A pinkish solid was obtained by dissolving the dipeptide in deionized, distilled water and lyophilizing, mp 90°C (decomp). ^{1}H NMR (300 MHz, CD_3OD): δ 0.85 (3H, t, J=7 Hz), 0.91 (3H, d, J=7 Hz), 1.10 (1H, m), 1.42 (1H, m), 1.81 (1H, m), 3.19 (1H, dd, J=7.3, 14.7 Hz), 3.36 (1H, dd, J=5, 16 Hz), 3.52 (1 H, d, J=5 Hz), 4.61 (1H, dd, J=5, 7 Hz), 6.97 (1H, ddd, J=1, 8, 8 Hz), 7.04 (1H, ddd, J=1, 8, 8 Hz), 7.11 (1H, bs), 7.28 (1H, d, J=8 Hz), 7.62 (1H, d, J=7 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 11.9, 15.5, 25.3, 29.3, 38.5, 57.0 (d, J_{C-C}=56 Hz), 59.8 (d, J_{C-C}=47 Hz), 112.2, 112.3, 119.7, 119.8, 124.5, 129.4, 138.1, 170.4, 177.6. IR (NaCl, CH_3OH): 3404, 3025, 2966, 1652, 1593, 1456, 1393, 742 cm^{-1}. $[\alpha]_D^{25}=+2.2°$ (c=0.005, CH_3OH).

4.3. Synthesis of [$^{13}C_2$]-L-tryptophanyl-L-isoleucine (26)

Prepared according to the route described in Scheme 8.

4.3.1. [1-^{13}C]-L-Isoleucine benzyl ester. [1-^{13}C]-L-Isoleucine (32.2 mg, 0.244 mmol), *p*-toluenesulfonic acid monohydrate (51 mg, 0.268 mmol), benzyl alcohol (100 μL), and dry toluene (15 mL) were refluxed for 24 h under an argon atmosphere through an addition funnel half filled with activated 4 Å molecular sieves. The toluene was evaporated under reduced pressure and the residue was covered with a layer of CH_2Cl_2. Saturated aqueous sodium carbonate was then added to make the solution basic. The aqueous layer was separated from the organic layer and extracted three more times with CH_2Cl_2. After drying over anhydrous sodium sulfate, the solvent was removed in

‡ The reported variable temperature ^{1}H- and ^{13}C NMR data were measured in the unlabeled compound. ^{13}C-labeled compound was identical by TLC mobility and ^{1}H- and ^{13}C NMR at 22°C.

vacuo to give a colorless oil. Yield: 34.4 mg, 63%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, J=7.3 Hz), 0.93 (3H, d, J=6.7 Hz), 1.19 (1H, m), 1.42 (1H, m), 1.55 (2H, bs, D_2O exch.), 1.78 (1H, m), 3.40 (1H, d, J=5 Hz), 5.14 (1H, d, J=12 Hz), 5.18 (1H, d, J=12 Hz), 7.36 (5H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.8, 16.0, 24.8, 39.3, 59.4 (d, J_{C-C}=57 Hz), 66.7, 128.5, 128.7, 136.0, 175.7. IR (NaCl, CH_2Cl_2): 3385, 2962, 1732, 1456, 1167 cm^{-1}. $[\alpha]_D^{25}$=+16.2° (c=0.015, CH_2Cl_2).

4.3.2. *N*-Benzyloxycarbonyl-[1-^{13}C]-L-tryptophan. [1-^{13}C]-L-Tryptophan (50.0 mg, 0.244 mmol) was protected in the same manner as described for *N*-benzyloxycarbonyl-[1-^{13}C]-L-isoleucine. Yield: 83.0 mg, 100%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.33 (2H, m), 4.75 (1H, dd, J=5, 12 Hz), 5.08 (1H, d, J=12 Hz), 5.14 (1H, d, J=12 Hz), 5.37 (1H, d, J=8 Hz, D_2O exch.), 6.90 (1H, s), 7.08 (1H, t, J=7 Hz), 7.19 (1H, t, J=7 Hz), 7.33, (6H, s), 7.57 (1H, d, J=8 Hz), 8.09 (1H, bs, D_2O exch.) 10.22 (1H, bs, D_2O exch.). ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.6, 54.5 (d, J_{C-C}=60 Hz), 67.1, 77.4, 109.9, 111.3, 118.6, 119.8, 122.2, 123.1, 127.6, 128.2, 128.3, 128.5, 136.0, 156.0, 176.5. IR (NaCl, CH_3OH): 3414, 2962, 1704, 1515, 1455, 1260, 1020, 795 cm^{-1}. $[\alpha]_D^{25}$=−4.0° (c=0.01, CH_3OH).

4.3.3. [$^{13}C_2$]-*N*-Benzyloxycarbonyl-L-tryptophanyl-L-isoleucine benzyl ester. The coupling of [1-^{13}C]-*N*-benzyloxycarbonyl-L-tryptophan (53.8 mg, 0.242 mmol) and [1-^{13}C]-L-isoleucine benzyl ester (81.4 mg, 0.239 mmol) was performed as described for [$^{13}C_2$]-*Z*-L-isoleucyl-L-tryptophan-benzyl ester. Yield: 123.0 mg, 99%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a white solid, mp 128–129°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, d, J=7.1 Hz), 0.79 (3H, t, J=7.3 Hz), 0.94 (1H, m), 1.23 (1H, m), 1.75 (1H, m), 3.15 (1H, dd, J=7.8, 14.5 Hz), 3.33 (1H, dd, J=5, 14.5 Hz), 4.51 (2H, m), 5.08 (2H, d, J=3 Hz), 5.13 (2H, s), 5.6 (1H, d, J=6 Hz, D_2O exch.), 6.22 (1H, d, J=8 Hz, D_2O exch.), 6.96 (1H, bs), 7.10 (1H, t, J=7 Hz), 7.18 (1H, ddd, J=1, 7, 7 Hz), 7.35 (11H, m), 7.69 (1H, d, J=7 Hz), 8.05 (1H, bs, D_2O exch.). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.7, 15.4, 25.2, 28.8, 38.0, 55.7 (d, J_{C-C}=55 Hz), 56.9 (d, J_{C-C}=61 Hz), 67.1, 67.2, 110.6, 111.4, 119.0, 120.0, 122.5, 123.6, 127.5, 128.3, 128.4, 128.6, 128.7, 128.8, 135.6, 136.5, 156.2, 171.3, 171.3. IR (NaCl, CH_2Cl_2): 3308, 3044, 2946, 1710, 1656, 1518, 1452, 1337, 1221, 1139, 738, 694 cm^{-1}. $[\alpha]_D^{25}$=+1.0° (c=0.01, CH_2Cl_2).

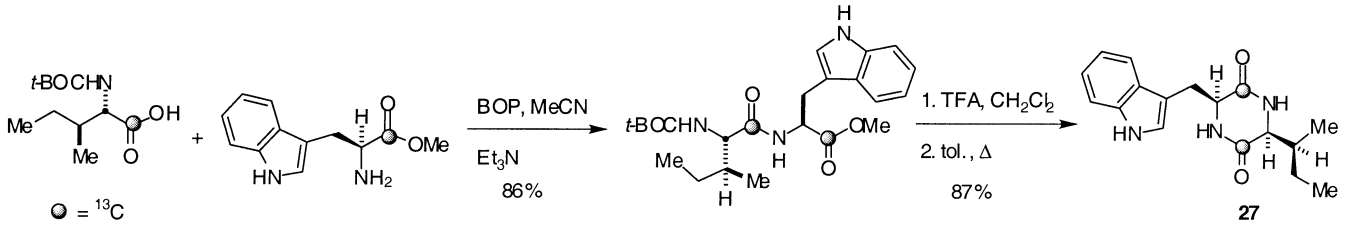
4.3.4. [$^{13}C_2$]-L-Tryptophanyl-L-isoleucine (26). [$^{13}C_2$]-*Z*-L-Tryptophanyl-L-isoleucine benzyl ester (48 mg, 0.0881 mmol) and 20% $Pd(OH)_2$/C (15 mg) were suspended in 2 mL of 95% ethanol. After bubbling argon through the reaction mixture, it was placed under $H_{2(g)}$ atmosphere with an $H_{2\ (g)}$ balloon. After 16 h TLC analysis showed the reaction to be complete. The reaction mixture was filtered through a pad of Celite and evaporated in vacuo. Yield: 30 mg, 100%. A white solid was obtained by dissolving the dipeptide in distilled deionized water and lyophilizing, mp 171°C (decomp). ^{1}H NMR (300 MHz, CD_3OD): δ 0.90 (3H, t, J=7 Hz), 0.92 (3H, d, J=7 Hz), 1.14 (1H, m), 1.55 (1H, m), 1.88 (1H, m), 3.17 (1H, dd, J=8, 15 Hz), 3.44 (1H, dd, J=5, 15 Hz), 4.15 (1H, dd, J=6, 6 Hz), 4.27 (1H, d, J=5 Hz), 7.04 (1H, ddd, J=1, 8, 8 Hz), 7.11 (1H, ddd, J=1, 7, 7 Hz), 7.21 (1H, bs), 7.35 (1H, d, J=8 Hz), 7.70 (1H, d, J=8 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 12.3, 16.5, 18.2, 26.3, 29.3, 39.2, 55.4 (d, J_{C-C}=53 Hz), 60.0 (d, J_{C-C}=60 Hz), 108.7, 112.6, 119.4, 120.3, 122.9, 125.8, 128.6, 138.4, 170.2, 175.9. IR (NaCl, CH_3OH): 3272, 3075, 2962, 1665, 1592, 1457, 1404, 742 cm^{-1}. $[\alpha]_D^{25}$=+6.6° (c=0.005, CH_3OH).

4.4. Synthesis of cyclo-L-isoleucine-L-tryptophan (27)

Prepared according to the route described in Scheme 9.

4.4.1. [1-^{13}C]-*N*-*t*-Butoxycarbonyl-L-isoleucine. NaOH (0.5 mL of a 1.0 M solution) was added to [1-^{13}C]-L-isoleucine (32.2 mg, 0.244 mmol, 90% ^{13}C) dissolved in dioxane (1.0 mL) and dd H_2O (0.5 mL). The solution was cooled in an ice bath while stirring and di-*tert*-butyl dicarbonate (62 μL, 0.270 mmol) was added. The reaction mixture was brought to room temperature and stirred for one hour. The dioxane was then evaporated under reduced pressure and the residue was covered with a layer of EtOAc and acidified to pH 2–3 with $KHSO_4$. The aqueous layer was separated from the organic layer and extracted two more times with EtOAc. The EtOAc fractions were pooled, dried over $MgSO_4$, and evaporated under reduced pressure to leave a colorless oil. Yield: 51.6 mg, 92%. ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.88 (3H, t, J=7.7 Hz), 0.91 (3H, d, J=6.6 Hz), 1.22 (1H, m), 1.42 (9H, s), 1.46 (1H, m), 1.80 (1H, m), 3.92 (1H, dd, J=7.7, 7.7 Hz), 6.04 (1H, bs), 10.29 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 15.5, 24.4, 28.3, 37.8, 57.8 (d, J_{C-C}=58 Hz), 80.0, 155.7, 177.2. IR (NaCl, CH_2Cl_2): 3326, 3110, 2971, 2587, 1715, 1663, 1509, 1454, 1401,1365, 1248, 1165 cm^{-1}. $[\alpha]_D^{25}$=+5.4° (c=0.012, CH_2Cl_2).

4.4.2. [1-^{13}C]-L-Tryptophan methyl ester. [1-^{13}C]-L-Tryptophan (50 mg, 0.244 mmol, 98% ^{13}C) and D, L-10-camphorsulfonic acid (115 mg, 0.495 mmol) were dissolved in 20 mL of absolute methanol. The solution was refluxed through an addition funnel containing 3 Å molecular sieves for 24 h under an argon atmosphere. The methanol was evaporated off under reduced pressure and the residue was



Scheme 9.

covered with a layer of EtOAc. Saturated aqueous sodium carbonate was then added to make the solution basic. The aqueous layer was separated from the organic layer and extracted 3 more times with EtOAc. After drying over anhydrous sodium sulfate, the solvent was removed in vacuo to leave a yellow oil. Yield: 41.3 mg, 78%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83 (2H, bs, D_2O exch.), 3.07 (1H, dd, J=7.6, 14.4 Hz), 3.31 (1H, dd, J=4.7, 14.4 Hz), 3.73 (3H, s), 3.86 (1H, dd, J=5, 7 Hz), 7.01 (1H, d, J=2 Hz), 7.14 (1H, ddd, J=1, 7, 7 Hz), 7.20 (1H, ddd, J=1, 7, 7 Hz), 7.34 (1H, d, J=8 Hz), 7.63 (1H, d, J=8 Hz), 8.52 (1H, bs, D_2O exch.). ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.9, 52.2, 55.1 (d, J_{C-C}=59 Hz), 111.0, 111.4, 118.8, 119.6, 122.2, 123.2, 127.6, 136.4, 175.9. IR (NaCl, CH_2Cl_2): 3387, 3162, 2936, 1732, 1586, 1448, 1201, 1099, 1007, 742 cm^{-1}. $[\alpha]_D^{25}$=+20.4° (c=0.01, CH_2Cl_2).

4.4.3. [$^{13}C_2$]-L-*N*-*t*-Butoxycarbonyl-isoleucyl-L-tryptophan methyl ester. A mixture of [^{13}C]-L-tryptophan methyl ester (41.3 mg, 0.180 mmol) and 41.4 mg (0.179 mmol) of [1-^{13}C]-*N*-*t*-butoxycarbonyl-L-isoleucine was dissolved in acetonitrile (2.75 mL). One equivalent of triethylamine (25 μL, 0.180 mmol) and BOP reagent (79.5 mg, 0.180 mmol) were added to the flask. After stirring for 2 h at room temperature, brine (10 mL) was added to the reaction mixture. The solution was extracted three times with EtOAc and the combined EtOAc layers were extracted with 2 M HCl, water, 0.5 M $NaHCO_3$, and brine successively. The organic layer was dried over $MgSO_4$, filtered, and evaporated to dryness. The crude product was purified by Chromatotron with a 9:1 mixture of CH_2Cl_2/CH_3OH. Yield: 60.0 mg, 80%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a white crystalline solid, mp 142–143°C. ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.82 (3H, t, J=7.3 Hz), 0.85 (3H, d, J=7.7 Hz), 1.10 (1H, m), 1.41 (9H, s), 1.44 (1H, m), 1.72 (1H, m), 2.89 (1H, bs), 3.17 (1H, dddd, J=6.6, 15.0, 15.0, 15.0 Hz), 3.59 (3H, s), 3.90 (1H, dd, J=6.6, 8.8 Hz), 4.66 (1H, dd, J=7.3, 14.3 Hz), 6.03 (1H, d, J=9.5 Hz), 6.99 (1H, ddd, J=1.1, 7.0 Hz), 6.08 (1H, ddd, J=1.1, 7.0, 7.0 Hz), 7.12 (1H, d, J=2.2 Hz), 7.35 (1H, d, J=7.7 Hz), 7.51 (1H, d, J=7.7 Hz), 7.70 (1H, d, J=7.0 Hz, 10.47 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 15.5, 24.8, 27.9, 28.5, 37.7, 52.7 (d, J_{C-C}=62 Hz), 59.3 (d, J_{C-C}=60 Hz), 80.0, 109.8, 111.5, 118.6, 119.8, 122.4, 123.3, 127.7, 136.4, 155.9, 171.5, 172.2. IR (NaCl, CH_2Cl_2): 3305, 2967, 1657, 1514, 1442, 1360, 1248, 1165 cm^{-1}. $[\alpha]_D^{25}$=+35.3° (c=0.01, CH_2Cl_2).

4.4.4. [$^{13}C]_2$-*cyclo*-L-Isoleucine-L-tryptophan (27). [$^{13}C_2$]-*N*-Boc-L-isoleucine-L-tryptophan-methyl ester (59 mg, 0.141 mmol) was dissolved in CH_2Cl_2 (10 mL). The solution was cooled to 0°C and trifluoroacetic acid (1 mL) was added. The reaction was stirred at 0°C under an argon atmosphere for 5 h. The solvent and most of the TFA were removed in vacuo. The residue was dissolved in dry toluene and refluxed under an argon atmosphere for 1 h. The toluene was evaporated in vacuo and the crude product was purified by means of flash column chromatography with a 9:1 mixture of CH_2Cl_2/CH_3OH to provide a white solid, mp 163°C (decomp). Yield: 37 mg, 87%. ^{1}H NMR (400 MHz, $CDCl_3$+5 drops CD_3OD): δ 0.76 (3H, t, J=7 Hz), 0.87 (3H, d, J=7 Hz), 0.91 (1H, m), 1.09 (1H, m), 1.83 (1H, m), 3.03 (1H, ddd, J=1, 9, 13 Hz), 3.50 (1H, ddd, J=3, 3, 14 Hz), 3.80 (1H, m), 4.21 (1H, m), 7.04 (1H, s), 7.06 (1H, t, J=7 Hz), 7.13 (1H, t, J=7 Hz), 7.32 (1H, d, J=8 Hz), 7.56 (1H, d, J=8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$+5 drops CD_3OD): δ 11.5, 14.9, 23.4, 30.4, 38.1, 54.9 (d, J_{C-C}=51 Hz), 59.8 (d, J_{C-C}=52 Hz), 108.8, 111.3, 118.4, 119.6, 122.2, 132.8, 126.8, 136.4, 166.9, 167.9. IR (NaCl, CH_3OH): 3319, 3198, 3045, 2957, 1666, 1457, 1326, 1095, 733 cm^{-1}. $[\alpha]_D^{25}$=−31.5° (c=0.0065, CH_3OH).

4.4.5. Synthesis of L-[1-^{13}C]-3(*S*)-methyl-proline (32). For the synthesis of **32**, essentially the procedure of Lavergne et al.[11] was used with the exception that a Hg° lamp was used in lieu of a Rayonnet lamp for the Hoffman–Loffler–Freytag reaction. In addition, *N*-Boc protection followed by flash column chromatography was used to purify the (*S*)-β-methyl-proline instead of PTLC of the free amino acid.

4.4.6. Synthesis of [1-^{13}C]-L-isoleucine ethyl ester (28). 7 equiv. of thionyl chloride (1.93 mL, 26.5 mmol) were added dropwise to a suspension of [1-^{13}C]-L-isoleucine (500 mg, 3.8 mmol) in 23 mL of absolute ethanol at 0°C. The mixture was brought to room temperature and stirred until all the solids dissolved and then refluxed for an additional 10 h. The reaction mixture was concentrated under reduced pressure, re-dissolved in water and made basic (pH=9–10) by the addition of 20% aqueous ammonia. The aqueous layer was extracted with EtOAc four times. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford a colorless oil. To remove residual EtOAc, the product was re-dissolved in CH_2Cl_2 and again concentrated under reduced pressure. Due to the volatility of the product, it should not be placed on a high vacuum line. Yield: 545 mg, 3.4 mmol, 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, t, J=7.3 Hz), 0.91 (3H, d, J=6.6 Hz), 1.15 (1H, m), 1.25 (3H, t, J=7.0 Hz), 1.40 (1H, m), 1.45 (2H, bs, D_2O exch.), 1.71 (1H, m), 3.30 (1H, d, J=5.1 Hz), 4.15 (2H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 14.2, 15.6, 24.6, 39.2, 59.0 (d, J_{C-C}=57 Hz), 60.4, 175.5. HRMS (FAB$^+$) calcd for $C_7{}^{13}C_1H_{18}O_2N_1$ (M+H) 161.1371, found 161.1373.

4.4.7. *N*-Cl-[1-^{13}C]-L-Isoleucine ethyl ester. To a solution of [1-^{13}C]-L-isoleucine ethyl ester (360.5 mg, 2.2 5 mmol) in benzene (2.8 mL) at 0°C was added one equivalent of freshly prepared *t*-butyl hypochlorite (228 μL, 2.25 mmol). The reaction mixture was kept below 5°C and stirred in the dark for 1.5 h. The mixture was washed with 1 mL of 0.1N HCl, and water (three times, 1 mL aliquots) before drying over anhydrous Na_2SO_4 and concentrating under reduced pressure at room temperature to afford a yellow oil. The product was carried on to the next step without further purification. Yield: 424 mg, 2.18 mmol, 97%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (3H, t, J=7.3 Hz), 0.92 (3H, d, J=5.1 Hz), 1.21 (1H, m), 1.30 (3H, t, J=7.7 Hz), 1.53 (1H, m), 1.72 (1H, m), 3.45 (1H, dd, J=6.6, 11.4 Hz), 4.25 (2H, q, J=7.0 Hz), 4.61 (1H, d, J=11.4 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.2, 14.3, 15.4, 5.8, 38.2, 61.2, 72.3 (d, J_{C-C}=57 Hz), 172.7.

4.4.8. *N*-Boc-[1-^{13}C]-3(*S*)-methyl-L-proline ethyl ester. A solution of [1-^{13}C]-*N*-Cl-L-isoleucine ethyl ester (424 mg,

2.18 mmol) in 85% H_2SO_4 (14.5 mL) at 0°C was prepared in a photochemical reaction vessel fitted with a quartz immersion well and Hg° lamp. After purging the system with argon for 15 min, the reaction mixture was then irradiated with a mercury lamp for 40 h under an atmosphere of argon while maintaining the temperature between 0–5°C. The reaction was tested for completion by taking an aliquot (5 drops) of the reaction mixture and treating with 5 mL of a 5% KI solution (in 1:1 water, acetone). When a clear pale yellow color was obtained the reaction was complete. After addition of 100 mL of an ice/water mixture, the reaction was carefully neutralized with aqueous 10 M NaOH at 0°C. The reaction mixture was concentrated under reduced pressure, the residue was taken up in absolute ethanol and the solids filtered off. Evaporation of the ethanolic solution under reduced pressure left crude L-[1-^{13}C]-3(*S*)-methyl-proline ethyl ester (298 mg, 1.87 mmol, 86%). The crude product was dissolved in 7.48 mL of a 1:1 mixture of dioxane and water and cooled to 0°C. Di-*tert*-butyl dicarbonate (449 mg, 2.06 mmol, 1.1 equiv.) and solid K_2CO_3 (258 mg, 1.87 mmol, 1 equiv.) were added. The reaction was slowly brought to room temperature and stirred for a total of 8 h. The dioxane was removed under reduced pressure, the reaction was lowered to a pH of 2 with 10% aqueous $KHSO_4$, and extracted four times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo to afford [1-^{13}C]-N-Boc-3(S)-methyl-L-proline ethyl ester (**31**) as an oil. Yield: 255 mg, 0.99 mmol (45% from of [1-^{13}C]-*N*-Cl-L-isoleucine ethyl ester). [1-^{13}C]-*N*-Boc-L-isoleucine ethyl ester was also recovered (50.3 mg, 0.19 mmol, 9% from of [1-^{13}C]-*N*-Cl-L-isoleucine ethyl ester). ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.13 (3H, d, J=7.0 Hz), 1.24 (3H, t, J=7.0 Hz), 1.40 (9H, s), 1.52 (1H, m), 2.01 (1H, m), 2.27 (1H, m), 3.33 (1H, m), 3.49 (1H, m), 3.72 (1H, d, J=5.5 Hz), 4.15 (2H, m). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 13.2, 17.4, 27.4, 31.0, 38.7, 44.9, 59.4, 65.4, 78.2, 152.6, 171.3. IR (NaCl, CH_2Cl_2): 2974, 2933, 2877, 1747, 1704, 14769, 1455, 1397, 1366, 1278, 1249, 1174, 1148, 1115, 1032 cm^{-1}. $[\alpha]_D^{25}$=−44.1° (c=1.24, CH_3OH).

4.4.9. L-[1-^{13}C]-3(*S*)-Methylproline. Compound **31** (62 mg, 0.24 mmol) was dissolved in 5 mL of anhydrous methanol. NaOH (48 mg, 1.2 mmol, 5 equiv.) was added and the solution was refluxed for 2 h. The methanol was removed in vacuo and 0.1 M HCl was added to achieve a pH=2. This mixture was extracted three times with EtOAc and the combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford [1-^{13}C]-*N*-Boc-3(*S*)-methyl-L-proline as a solid. Yield: 50 mg, 0.217 mmol, 90%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a solid with mp 147–148°C. ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.12 (3H, d, J=7.0 Hz), 1.40 (9H, s) 1.48 (1H, m), 1.99 (1H, m), 2.28 (1H, m), 3.32 (1H, m), 3.47 (1H, m), 3.66 (1H, d, J=5.5 Hz), 11.77 (1H, bs). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 17.7, 27.5, 31.0, 38.7, 44.8, 65.4, 152.8, 172.6. IR (NaCl, CH_2Cl_2): 3438 (broad), 3106 (broad), 2965, 2604 (broad), 1694, 1413, 1252, 1157 cm^{-1}. $[\alpha]_D^{25}$=−135° (c=0.1, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{10}{}^{13}C_1H_{20}O_4N_1$ (M+H) 231.1426, found 231.1420.

[1-^{13}C]-*N*-Boc-3(*S*)-methyl-L-proline was taken up in 0.5 mL of CH_2Cl_2 and placed under argon while stirring at 0°C. TFA (348 μL, 4.34 mmol, 20 equiv.) was added slowly to this solution. The resulting solution was allowed to come to room temperature while continuing to stir over 3 h. The CH_2Cl_2 and excess TFA were removed in vacuo. The crude reaction mixture was loaded onto a Dowex 50X2-100 ion exchange column and washed with 500 mL of water until the pH of the eluate was neutral. The free amino acid was then eluted with 2% NH_4OH. The NH_4OH solution was evaporated under reduced pressure. The residue was then taken up in a small amount of distilled deionized water and lyophilized to leave [1-^{13}C]-3(*S*)-methyl-L-proline (**32**) as an off-white amorphous solid. Yield: 28 mg, 0.215 mmol, 99%. ^{1}H NMR (300 MHz, D_2O): δ 1.23 (1H, d J=7.0 Hz), 1.68 (1H, m), 2.20 (1H, m), 2.39 (1H, m), 3.39 (2H, m), 3.60 (1H, dd, J=4.0, 6.7 Hz). ^{13}C NMR (75 MHz, D_2O): δ 13.1, 27.7, 33.7, 40.5, 62.6 (d, J_{C-C}=53 Hz), 169.8. Enhanced ^{13}C peak. IR (NaCl, CH_3OH): 3389–2284, 2958, 1556, 1434, 1378, 1299, 1282, 1212, 1122, 1030, 961 cm^{-1}. $[\alpha]_D^{25}$=−27° (c=0.1, H_2O). (Literature: $[\alpha]_D^{25}$=−29°±2° (c=0.1, H_2O)) HRMS (FAB$^+$) calcd for $C_5{}^{13}C_1H_{12}O_2N_1$ (M+H) 131.0902, found 131.0907.

4.5. Synthesis of [$^{13}C_2$]-*cyclo*-L-tryptophan-3(*S*)-methyl-L-proline (35)

4.5.1. [$^{13}C_2$]-*N*-Boc-L-tryptophan-3(*S*)-methyl-L-proline ethyl ester. [1-^{13}C]-L-Tryptophan (60 mg, 0.292 mmol) was dissolved in 1.17 mL of a 1:1 mixture of dioxane and water and cooled to 0°C under an inert atmosphere. Di-*tert*-butyl dicarbonate (76.1 μL, 0.331 mmol, 1.1 equiv.) and anhydrous K_2CO_3 (40.3 mg, 0.292 mmol, 1 equiv.) were added to the stirring solution. The reaction mixture was brought to room temperature and stirred for 3 h. The dioxane was removed under reduced pressure, the reaction mixture was lowered to pH=2 with 10% aqueous $KHSO_4$, and extracted four times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo to afford [1-^{13}C]-*N*-Boc-L-tryptophan (**34**). In a separate reaction vessel, [1-^{13}C]-*N*-Boc-3(*S*)-methyl-L-proline ethyl-ester (**31**, 75.4 mg, 0.292 mmol) was taken up in 0.5 mL of CH_2Cl_2, placed under argon and stirred at 0°C. To this solution, TFA (469 μL, 5.84 mmol, 20 equiv.) was added slowly. The solution was allowed to come to room temperature while being stirred for 3 h. The CH_2Cl_2 and excess TFA were removed in vacuo to leave the [1-^{13}C]-3(*S*)-methyl-L-proline ethyl ester TFA salt as an oil (**33**). The crude protected amino acids were combined with one equivalent of BOP reagent (129 mg, 0.292 mmol) and 2 equiv. of triethylamine (81.4 μL, 0.584 mmol) in 438 μL of dry acetonitrile. The reaction was stirred for 3.5 h at room temperature under an argon atmosphere. A saturated aqueous solution of NaCl was added and the mixture was extracted three times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was purified by means of flash column chromatography (eluted with 50% EtOAc/hexane) to provide [$^{13}C_2$]-*N*-Boc-L-tryptophan-L-3(*S*)-methyl-proline ethyl ester as a foam. An analytical sample of unlabeled material was recrystallized from EtOAc and hexane to provide a white solid, melting point 152.5°C. Yield: 101.8 mg,

0.229 mmol, 78% (2 steps). ^{1}H NMR (300 MHz, d$_6$-DMSO, 120°C):[§] δ 1.12 (3H, br s), 1.21 (3H, t, *J*=7.3 Hz), 1.34 (10H, s), 1.57 (1H, m), 2.08 (1H, br s), 2.23 (1H, br s), 2.87–3.37 (4H, m), 3.86 (1H, m), 4.13 (2H, dd, *J*=7.0, 14.3 Hz), 4.54 (1H, br s), 6.11 (1H, br s), 7.00 (1H, dd, *J*=1.1, 7.0 Hz), 7.08 (1H, dd, *J*=1.1, 7.0 Hz), 7.35 (1H, d, *J*=8.0 Hz), 7.56 (1H, d, *J*=8.0 Hz), 10.48 (1H, br s). ^{13}C NMR (75 MHz, d$_6$-DMSO, 120°C): δ 13.2, 17.1, 26.8, 32.1, 36.4, 45.2, 52.4 (d, J_{CC}=55 Hz), 59.5, 65.3 (d, J_{CC}=60 Hz), 77.7, 109.2, 110.6, 117.3, 117.7, 120.2, 123.1, 127.0, 135.8, 154.1, 170.0, 170.5. IR (NaCl, CH_2Cl_2): 3323, 3057, 2966, 2926, 2873, 1738, 1711, 1648, 1510, 1455, 1391, 1366, 1351, 1297, 1255, 1170, 1098, 1030, 955, 863, 742 cm^{-1}. $[\alpha]_D^{25}$=−18.9° (*c*=1.2, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{22}{}^{13}C_2H_{34}O_5N_3$ (M+H) 446.2566, found 446.2574.

4.5.2. [$^{13}C_2$]-*cyclo*-L-Tryptophan-L-3(*S*)-methylproline (35). To a stirred solution of [$^{13}C_2$]-*N*-Boc-L-tryptophan-3(*S*)-methyl-L-proline ethyl ester (102 mg, 0.229 mmol) at 0°C under an inert atmosphere, TFA (353 μL, 4.58 mmol, 20 equiv.) was slowly added. The solution was brought to room temperature and was stirred for 3 h. The CH_2Cl_2 and excess TFA were removed in vacuo. The residue was taken up in aqueous 10% $NaCO_3$ and extracted three times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 1.5 mL of toluene, 2-hydroxypyridine (4.4 mg, 0.046 mmol, 0.2 equiv.) was added, and the solution was refluxed under an argon atmosphere for 4 h. The toluene was removed under reduced pressure and the product purified by means of flash column chromatography (eluted with 2% CH_3OH/CH_2Cl_2) to provide [$^{13}C_2$]-*cyclo*-L-tryptophan-3(*S*)-methyl-L-proline (**35**) as a white solid. Yield: 42 mg, 0.140 mmol, 61%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (3H, d *J*=6.6 Hz), 1.53 (1H, m), 2.13 (1H, m), 2.34 (1H, m), 2.94 (1H, ddd, *J*=2.3, 10.9, 14.8 Hz), 3.55 (3H, m), 3.73 (1H. d, *J*=14.7 Hz), 4.32 (1H, m), 5.63 (1H d, *J*=4.3 Hz), 7.09 (1H, d, *J*=2.0 Hz), 7.12 (1H, ddd, *J*=0.8, 7.8, 7.8 Hz), 7.22 (1H, ddd, *J*=1.2, 8.2, 8.2 Hz), 7.38 (1H, d, *J*=8.2 Hz), 7.57 (1H, d, *J*=8.5 Hz), 8.19 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.2, 26.8, 31.6, 37.1, 44.1, 54.2 (d, J_{CC}=52 Hz), 64.0 (d, J_{CC}=52 Hz), 109.9(d, J_{CC}=29 Hz), 111.5, 118.5, 120.0, 122.8, 123.2, 126.7, 136.6, 165.6, 169.4. IR (NaCl, CH_2Cl_2): 3389, 3054, 2922, 2851, 1632, 1612, 1454, 1415, 1301, 1102, 737, 693, 673 cm^{-1}. $[\alpha]_D^{25}$=−92.9° (*c*=1.2, 5% CH_3OH/CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{15}{}^{13}C_2H_{20}O_2N_3$ (M+H) 300.1623, found 300.1617.

4.6. Synthesis of [α-^{15}N, 1-^{13}C]-2-(1,1-dimethyl-2-propenyl)-L-tryptophan (37)

4.6.1. [2-^{15}N, 1-^{13}C]-2-Amino-*N*-bismethylsulfanyl-ethene-3-[2-(1,1-dimethyl-allyl)-1*H*-indol-3-yl]-1-(10,10-dimethyl-3,3-dioxo-3-thia-4-aza-tricyclo[5.2.1.0^{0,0}]dec-4-yl)-propan-1-one (42). The [^{13}C, ^{15}N]-labeled Oppolzer sultam glycinate (**40**, 330 mg, 0.887 mmol), the gramine derivative (**6**, 255 mg, 1.05 mmol, 1.2 equiv.), and tri-*n*-butylphosphine (66 μL, 0.263 mmol, 0.3 equiv.) were refluxed in acetonitrile (7.9 mL, 0.11 M) under argon for 8 h. After cooling to room temperature, the solvent was concentrated under reduced pressure and the crude product was purified by means of flash silica gel column chromatography (20% EtOAc/hex.) to give 361 mg, 0.630 mmol of compound **42** (72% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.76 (3H, s), 0.86 (3H, s), 1.27(2H, m), 1.55 (1H, m), 1.56 (3H, s), 1.61 (3H, s), 1.65 (1H, dd, *J*=3.9, 3.9 Hz), 1.80 (2H, m), 1.89 (1H, dd, *J*=7.8, 13.7 Hz), 2.25 (3H, s), 2.36 (3H, s), 3.32 (1H, d, *J*=13.7 Hz), 3.36 (1H, m), 3.39 (1H, d, *J*=14.0 Hz), 3.48 (1H, m), 3.83 (1H, dd, *J*=5.0, 7.0 Hz), 5.11 (1H, dd, *J*=1, 10.6 Hz), 5.15 (1H, dd, *J*=0.8, 17.6 Hz), 5.54 (1H, m), 6.19 (1H, dd, *J*=10.6, 17.6 Hz), 7.01(1H, ddd, *J*=1.2, 7.0, 7.0 Hz), 7.05 (1H, ddd, *J*=1.6, 7.0, 7.0 Hz), 7.16 (1H, dd, *J*=1.6, 6.6 Hz), 7.74 (1H, dd, *J*=2.0, 7.0 Hz), 7.79 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.8 (d, J_{N-C}=5 Hz), 15.9, 19.8, 20.4, 26.4, 27.6, 27.7, 29.8, 32.8, 38.2, 39.5, 44.6, 47.5, 48.1, 53.2, 65.3, 66.7 (d, J_{C-C}=58 Hz), 105.7 (d, J_{N-C}=3 Hz), 109.6, 111.6, 118.9, 120.1, 121.2, 129.9, 134.1, 140.6, 146.5, 161.7, 171.9 (d, J_{N-C}=3 Hz). IR (NaCl, CH_2Cl_2): 3411, 3081, 3055, 2961, 2926, 2883, 1653, 1558, 1541, 1457, 1335, 1207, 1133, 1049, 1009, 912, 733 cm^{-1}. Optical rotation: $[\alpha]_D$=+7.5° (CH_3OH, *c*=0.53). HRMS (FAB$^+$) calcd for $C_{28}{}^{13}C_1H_{40}N_1{}^{15}N_1O_3S_3$: 576.2236. Found 576.2243 (M+H).

4.6.2. [α-^{15}N, 1-^{13}C]-*N*-bismethylsulfanyl-ethene-2-(1,1-dimethyl-2-propenyl)-L-tryptophan (43). Solid LiOH (14 mg, 0.59 mmol, 5.0 equiv.) was added to a THF/water (2:1) solution of **42** (67.5 mg, 0.118 mmol, 0.03 M). The mixture was stirred at ambient temperature for 24 h under an atmosphere of argon. The THF was removed under reduced pressure and the remaining aqueous layer was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting with dichloromethane three times. The product was purified by means of flash column chromatography (eluted with 2% CH_3OH/CH_2Cl_2) to provide 37.5 mg of compound **43** (84% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (3H, s), 1.56 (3H, s), 2.18 (3H, s), 2.40 (3H, s), 3.29 (1H, m), 3.59 (1H, dddd, *J*=2.7, 3.5, 3.5, 14.8 Hz), 4.78 (1H, m), 5.16 (1H, d, *J*=10.5 Hz), 5.18 (1H d, *J*=17.6 Hz), 6.12 (1H, dd, *J*=10.5, 17.2 Hz), 7.03 (1H, ddd, *J*=0, 7.8, 7.8 Hz), 7.09 (1H, ddd, *J*=1.2, 7.0, 7.0 Hz), 7.23 (1H, d, *J*=7.0 Hz), 7.52 (1H, d, *J*=7.8 Hz), 7.88 (1H, br s), 10.02 (1H, very br s). ^{13}C NMR (300 MHz, $CDCl_3$): δ 14.9 (J_{N-C}=5 Hz), 15.4, 27.6, 27.7, 29.6, 39.2, 66.4 (d, J_{C-C}=58 Hz), 106.4, 110.1, 112.1, 118.6, 119.1, 121.3, 130.0, 134.1, 140.4, 145.8, 170.0, 173.0 (J_{N-C}=3 Hz). IR (NaCl, CH_2Cl_2): 3374, 3054, 2923, 2852, 1667, 1547, 1461, 1428, 1307, 1242, 1009, 913, 742 cm^{-1}. $[\alpha]_D^{25}$=+16.4° (*c*=0.495, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{18}{}^{13}C_1H_{25}N_1{}^{15}N_1O_2S_2$: 379.1361. Found 379.1353 (M+H).

4.6.3. [α-^{15}N, 1-^{13}C]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophan (37). Compound **43** (37.5 mg, 0.099 mmol) was stirred with 1 M HCl (0.99 mL, 10 equiv.) in THF (1.8 mL, 0.05 M) at room temperature for 24 h under an argon atmosphere. The THF was removed under reduced pressure and the remaining aqueous layer was extracted with diethyl ether. The diethyl ether layer was washed twice with a small amount of water and the combined aqueous layers were extracted twice with diethyl ether. The aqueous layer was evaporated under reduced pressure, re-dissolved in a small amount of distilled water and loaded onto a Dowex H$^+$ column. The column was washed several

[§] The observed peaks were still broad at 120°C in DMSO-d$_6$.

times with distilled water before eluting with approximately 200 mL of 3% aqueous NH_4OH. The eluate was evaporated in vacuo and then re-suspended in a small amount of de-ionized water. The resulting solution was lyophilized to give compound **37** as an off-white solid in 59% yield (16 mg, 0.058 mmol). ^{1}H NMR (400 MHz, CD_3OD): δ 1.55 (3H, s), 1.57 (3H, s), 3.12 (1H, m), 3.58 (1H, m), 3.88 (1H, m), 4.11 (1H, dd, J=1.2, 10.5 Hz), 5.14 (1H, dd, J=0.8, 17.6 Hz), 6.19 (1H, dd, J=10.9, 17.6 Hz), 6.99 (1H, ddd, J=0, 7.0, 7.8 Hz), 7.05 (1H, ddd, J=0.8, 7.0, 7.8 Hz), 7.32 (1H, d, J=8.2 Hz), 7.59 (1H, d, J=7.8 Hz). ^{13}C NMR (75 MHz, CD_3OD): 28.7, 29.0, 40.4, 58.0 (dd, J_{N-C}=5 Hz, J_{C-C}=54 Hz), 105.7 (J_{N-C}=3 Hz), 112.1, 112.4, 118.9, 120.3, 122.3, 130.7, 136.8, 143.1, 147.9, 175.4. IR (neat, NaCl): 3689–1738 (broad), 3349, 2966, 2924, 1631, 1538, 1461, 1381, 1334, 1302, 919, 745 cm^{-1}. $[\alpha]_D$=+7.5° (CH_3OH, c=0.53). HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{21}N_1{}^{15}N_1O_2$, 275.1607. Found 275.1594 (M+H).

4.7. Synthesis of [$^{13}C_2$]-*cyclo*-2-(1,1-dimethyl-2-propenyl)-L-tryptophan-3(*S*)-methyl-L-proline (36)

4.7.1. [1-^{13}C]-2-Amino-*N*-bismethylsulfanyl-ethene-3-[2-(1,1-dimethyl-allyl)-1*H*-indol-3-yl]-1-(10,10-dimethyl-3,3-dioxo-3-thia-4-aza-tricyclo[5.2.1.0^{0,0}]dec-4-yl)-propan-1-one (45). Preparation of **45** from **44** (190 mg, 0.508 mmol) was carried out in the same manner as **42** from **40**. Yield: 249 mg, 0.435 mmol, 86%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78 (3H, s), 0.85 (3H, s), 1.25 (2H, m), 1.55 (1H, m), 1.57 (3H, s), 1.61 (3H, s), 1.65 (1H, dd, J=3.5, 3.5 Hz), 1.78 (2H, m), 1.89 (1H, dd, J=7.8, 13.7 Hz), 2.24 (3H, s), 2.36 (3H, s), 3.30 (1H, d, J=13.7 Hz), 3.37 (1H, m), 3.38 (1H, d, J=13.7 Hz), 3.49 (1H, ddd, J=1.6, 6.5, 14.0 Hz), 3.83 (1H, dd, J=4.7, 7.4 Hz), 5.11 (1H, d, J=10.9 Hz), 5.15 (1H, d, J=18.8 Hz), 5.56 (1H, dd, J=7.8, 7.8 Hz), 6.20 (1H, dd, J=10.6, 17.6 Hz), 7.03 (2H, m), 7.17 (1H, d, J=8.2 Hz), 7.75 (1H, d, J=7.0 Hz), 7.89 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.7, 15.7, 19.6, 20.3, 26.2, 27.5, 27.6, 29.7, 32.6, 38.0, 39.4, 44.5, 47.4, 48.0, 53.0, 65.1, 66.6 (d, J_{CC}=57 Hz), 105.5, 109.5, 111.4, 118.7, 119.9, 121.0, 129.7, 134.1, 140.5, 146.4, 161.4 (d, J_{CC}=6 Hz), 173.5. IR (NaCl, CH_2Cl_2): 3411, 3081, 3055, 2961, 2926, 2883, 1653, 1558, 1541, 1457, 1335, 1207, 1133, 1049, 1009, 912, 733 cm^{-1}. HRMS (FAB+): Calcd for $C_{28}{}^{13}C_1H_{40}N_3O_3S_3$ 575.2265. Found 575.2264 (M+H).

4.7.2. [1-^{13}C]-*N*-Bismethylsulfanyl-ethene-2-(1,1-dimethyl-2-propenyl)-L-tryptophan (46). The preparation of **46** from **45** (40 mg, 0.070 mmol) was achieved in the same manner as **43** from **42**. Yield: 23 mg, 0.061 mmol, 87%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (3H, s), 1.56 (3H, s), 2.18 (3H, s), 2.40 (3H, s), 3.29 (1H, ddd, J=2.7, 10.1, 14.4 Hz), 3.37 (1H, ddd, J=2.7, 2.7, 14.8 Hz), 4.79 (1H, ddd, J=3.9, 3.9, 8.2 Hz), 5.16 (1H, dd, J=1.2, 10.5 Hz), 5.19 (1H dd, J=0, 17.6 Hz), 6.12 (1H, dd, J=10.5, 17.1 Hz) 7.03 (1H, ddd, J=1.2, 8.2, 8.2 Hz), 7.09 (1H, ddd, J=1.2, 7.1, 7.1 Hz), 7.23 (1H, d, J=8.2 Hz), 7.52 (1H, d, J=7.4 Hz), 7.88 (1H, br s), 10.02 (1H, very br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.9, 15.4, 27.6, 27.7, 29.6, 39.2, 66.4 (d, J_{CC}=56 Hz), 66.4, 106.4, 110.1, 112.1, 118.6, 119.1, 121.3, 130.0, 134.1, 140.4, 145.8, 173.5 (enhanced ^{13}C peak). IR (NaCl, CH_2Cl_2): 3374, 3054, 2923, 2852, 1667, 1547, 1461, 1428, 1307, 1242, 1009, 913, 742 cm^{-1}. $[\alpha]_D{}^{25}$=+16.4° (c=0.495, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{18}{}^{13}C_1H_{25}N_2O_2S_2$ 378.1391. Found 378.1378 (M+H).

4.7.3. [1-^{13}C]-*N*-Bismethylsulfanyl-ethene-2-(1,1-dimethyl-2-propenyl)-L-tryptophanyl-3(*S*)-methyl-L-proline ethyl ester (47). *N*-Boc-3(*S*)-methyl-L-proline ethyl ester (**31**, 57.4 mg, 0.222 mmol) was taken up in 0.5 mL of CH_2Cl_2, placed under argon and stirred at 0°C. TFA (469 μL, 5.84 mmol, 20 equiv.) was then added slowly. The solution was brought to room temperature and was stirred an additional 3 h. The CH_2Cl_2 and excess TFA were removed in vacuo to leave the [1-^{13}C]-3(*S*)-methyl-L-proline ethyl ester TFA salt (**33**) as an oil. To **33** was admixed **46** (84 mg, 0.222 mmol) with 1 equiv. of BOP reagent (98 mg, 0.222 mmol) and 2 equiv. of triethylamine (68 μL, 0.488 mmol) in 3.4 mL of dry acetonitrile. The reaction was stirred for 3.5 h at room temperature under an argon atmosphere. A saturated aqueous solution of NaCl was added and the mixture was extracted three times with EtOAc. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified via flash column chromatography (eluted with 4% CH_3OH/CH_2Cl_2) to afford **47**. Yield: 106.8 mg, 0.206 mmol, 93% (2 steps). ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.10 (3H, br s), 1.21 (3H, t, J=7.3 Hz), 1.56 (6H, s), 2.65 (1H, br s), 2.28 (2H, m), 2.88 (6H, s), 3.38 (2H, br s), 3.56 (1H, br s), 3.76 (1H, br s), 3.88(1H, dd, J=4.8, 4.8 Hz), 4.12 (2H, dddd, J=2.9, 7.0, 7.0, 7.0 Hz), 4.90 (1H, br s), 5.05 (1H, dd, J=1.1, 10.6 Hz), 5.08 (1H dd, J=1.1, 17.6 Hz), 6.26 (1H, dd, J=10.3, 17.2 Hz), 6.91 (1H, ddd, J=1.1, 7.3, 7.3 Hz), 6.99 (1H, ddd, J=1.1, 7.0, 7.0 Hz), 7.29 (1H, d, J=7.7 Hz), 7.46 (1H, d, J=7.3 Hz), 9.98 (1H, br s). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 13.2, 13.9, 17.4, 27.3, 27.4, 28.1, 32.0, 38.4, 45.2, 59.5, 67.0 (d, J_{CC}=57.7 Hz), 109.9, 110.1 117.3, 117.6, 119.6, 129.1, 134.4, 140.2, 146.1, 167.0, 169.0, 170.7. IR (NaCl, CH_2Cl_2): 3319, 3052, 2966, 2927, 2874, 1699, 1598, 1577, 1460, 1429, 1312, 1235, 1174, 1154, 1032, 1008, 912, 742 cm^{-1}. $[\alpha]_D{}^{25}$=−74.9° (c=0.595, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{25}{}^{13}C_2H_{38}N_3O_3S_2$ 518.2422. Found 518.2422 (M+H).

4.7.4. [$^{13}C_2$]-*Cyclo*-2-(1,1-dimethyl-2-propenyl)-L-tryptophan-3(*S*)-methyl-L-proline (36). Compound **47** (57 mg, 0.113 mmol) was stirred with 1 M HCl (1.13 mL, 10 equiv.) in THF (2.26 mL, 0.05 M) at room temperature for 24 h. Enough aqueous solution of 10% $NaCO_3$ was added to turn the pH basic and the reaction was extracted three times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 2 mL of toluene, 2-hydroxypyridine (2.0 mg, 0.023 mmol, 0.2 equiv.) was added, and the solution refluxed under an argon atmosphere for 4 h. The toluene was removed under reduced pressure and the product purified by means of flash column chromatography (eluted with 4% CH_3OH/CH_2Cl_2) to provide **36** as a white solid which was recrystallized from ethyl acetate and hexane. Yield: 40 mg, 0.109 mmol, 97%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (3H, d, J=6.24 Hz), 1.54 (6H, s), 2.16 (1H, m), 2.39 (1H, m), 3.15 (1H, ddd, J=1.6, 11.3, 14.4 Hz), 3.54 (2H, m), 3.61 (1H, ddd, J=1.9, 9.4, 9.4 Hz), 3.68 (1H, dd, J=3.9, 15.2 Hz), 4.38 (1H, d,

J=11.7 Hz), 5.16 (1H, dd, J=0, 17.9 Hz), 5.17 (1H dd, J=0, 10.9 Hz), 5.6 (1 H, br s), 6.26 (1H, dd, J=10.9, 17.9 Hz), 7.08 (1H, t, J=7.4 Hz), 6.99 (1H, t, J=7.0 Hz), 7.30 (1H, d, J=8.2 Hz), 7.46 (1H, d, J=8.2 Hz), 8.02 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.2, 26.0, 27.9, 28.0, 31.6, 37.2, 39.0, 44.1, 54.8 (d, J_{CC}=52.2 Hz), 64.0 (d, J_{CC}=53.7 Hz), 104.7, 110.8, 112.8, 117.9, 120.1, 122.2, 129.1, 134.3, 141.4, 145.6, 165.9, 169.3. IR (NaCl, CH_2Cl_2): 3364, 3051, 2969, 2867, 1633, 1461, 1403, 1321, 1305, 1252, 1223, 1009, 921, 743 cm^{-1}. $[\alpha]_D^{25}$=−30° (c=0.07, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{20}{}^{13}C_2H_{27}N_3O_2$ 367.2170. Found 367.2185 (M+H).

4.7.5. [$^{13}C_2$]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophanyl-3(*S*)-methyl-L-proline (38). Five equivalents of LiOH (6.8 mg, 0.285 mmol) were added to a 0.03 M solution of **47** (29.5 mg, 0.057 mmol) in THF/H_2O (2:1). The mixture was stirred at room temperature for 24 h. The THF was removed in vacuo and the residue was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting three times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was re-suspended in THF (1.14 mL), 1N HCl was added (0.57 mL), and the mixture was stirred for 24 h at room temperature. The THF and excess HCl were removed under reduced pressure. The residue was loaded onto a suitably prepared Dowex 50WX2-100 ion exchange column, then desalted with de-ionized water and eluted with 2% aqueous NH_4OH. The eluate was evaporated in vacuo and then re-suspended in a small amount of de-ionized water lyophilized to give a white solid (22 mg, 0.057 mmol, 100% yield). ^{1}H NMR (400 MHz, D_2O): δ 0.12 (3H, d, J=6.6 Hz), 0.49 (1H, m), 1.53 (1H, m), 1.56 (3H, s), 1.60 (3H, s), 2.00 (1H, m), 2.63 (1H, dd, J=0, 3.0 Hz), 3.08 (1H, m), 3.14 (1H, dd, J=0, 14.4 Hz), 3.30 (2H, m), 3.84 (1H, dd, J=0, 9.4 Hz), 5.21 (1H dd, J=0, 10.9 Hz), 5.27 (1H, dd, J=0.8, 17.2 Hz), 6.28 (1H, dd, J=10.9, 17.2 Hz), 7.10 (1H, t, J=7.4 Hz), 7.18 (1H, t, J=7.0 Hz), 7.40 (1H, d, J=8.2 Hz), 7.42 (1H, d, J=9.0 Hz). ^{13}C NMR (100 MHz, D_2O): δ 17.8, 26.9, 27.1, 29.3, 29.4, 38.9, 39.0, 46.4, 52.8 (d, J_{C-C}=52 Hz), 68.9 (d, J_{C-C}=54 Hz), 103.8, 111.4, 111.9, 117.6, 119.4, 121.6, 129.0, 134.5, 142.2, 146.0, 173.3, 178.7. IR (NaCl, CH_2Cl_2): 3566–1600 (broad), 3421, 3355, 3056, 2965, 2930, 2873, 1558, 1458, 1362, 921, 743 cm^{-1}. $[\alpha]_D^{25}$=+32° (c=0.1, H_2O). HRMS (FAB$^+$) calcd for $C_{20}{}^{13}C_2H_{30}N_3O_3$ 386.2354. Found 386.2347 (M+H).

4.7.6. [1-^{13}C]-2-(1,1-dimethyl-2-propenyl)-L-tryptophan (48). Compound **48** was synthesized from compound **44** in 66% yield with the procedure described for the preparation of **37** from **43**. ^{1}H NMR (400 MHz, CD_3OD): δ 1.57 (3H, s), 1.59 (3H, s), 2.99 (1H, dd, J=0, 12.5 Hz), 3.37 (1H, m), 3.68(1H, m), 5.08 (1H, d, J=0, 10.5 Hz), 5.09 (1H, dd, J=0, 17.5 Hz), 6.20 (1H, dd, J=10.5, 17.5 Hz), 7.03 (1H, t, J=7.0 Hz), 7.03 (1H, t, J=7.0 Hz), 7.30 (1H, d, J=7.8 Hz), 7.65 (1H, d, J=7.7 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 28.9, 28.0, 33.1, 40.5, 58.8 (J_{C-C}=55 Hz) 108.2, 111.8, 111.9, 119.7, 119.8, 122.0, 131.1, 136.7, 142.5, 148.3, 182.3. IR (neat, NaCl): 3349, 2966, 2924, 1631, 1538, 1461, 1381, 1334, 1302, 919, 745 cm^{-1}. $[\alpha]_D^{25}$=+11.5° (H_2O, c=0.23). HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{21}N_2O_2$: 274.1637. Found 274.1643 (M+H).

4.7.7. [1-^{13}C]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophan methyl ester (49). [1-^{13}C]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophan (**48**, 51 mg, 0.187 mmol) and D,L-camphorsulfonic acid (115 mg, 0.497 mmol, 2.0 equiv.) were dissolved in 2.5 mL of anhydrous methanol. Under an argon atmosphere, the resulting solution was refluxed for 24 h through an addition funnel containing activated 3 Å molecular sieves. The methanol was removed under reduced pressure and the residue was covered with a layer of ethyl acetate. The solution was made basic by the addition of aqueous saturated sodium carbonate. The aqueous layer was separated from the organic and then extracted three times with ethyl acetate. The organic extracts were pooled together and dried over anhydrous sodium sulfate. Finally, the solvent was removed in vacuo to afford **49** (53.5 mg, 0.186 mmol, 99% yield) as a yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62 (6 H, s), 1.72 (2H, br s), 3.12 (1H, dd, J=9.5, 14.3 Hz), 3.39 (1H, dd, J=5.1, 14.7 Hz), 3.73 (3H, s), 3.94 (1H, dd, J=4.8, 9.5 Hz), 5.22 (1H, dd, J=1.1, 10.6 Hz), 5.23 (1H, dd, J=1.1, 17.6 Hz), 6.19 (1H, dd, J=10.3, 17.6 Hz), 7.12 (1H, ddd, J=1.1, 7.0 Hz), 7.18 (1H, dd, J=1.1, 7.0 Hz), 7.33 (1H, dd, J=0.7, 7.0 Hz), 7.6 (1H, dd, J=0.7, 7.7 Hz), 7.98 (1H, br s). ^{13}C NMR (100 MHz, CD_3OD): δ 27.8, 27.9, 31.2, 51.9 (d, J_{C-C}=3 Hz), 55.4 (d, J_{C-C}=54 Hz), 56.1, 106.7, 110.4, 112.0, 118.4, 119.2, 121.4, 129.6, 134.1, 140.4, 145.9, 180.0. IR (neat, NaCl): 3396, 3284, 3081, 3056, 2962, 2924, 2853, 1699, 1653, 1457, 1436, 1260, 1199, 1154, 1095, 1018, 919, 862, 799, 742 cm^{-1}. $[\alpha]_D^{25}$=−16.5° (CH_2Cl_2, c=0.085). HRMS (FAB+): Calcd for $C_{16}{}^{13}C_1H_{23}N_2O_2$: 288.1793. Found 288.1797 (M+H).

4.7.8. [$^{13}C_2$]-*N*-Boc-3(*S*)-methyl-L-proline-2-(1,1-dimethyl-2-propenyl)-L-tryptophan methyl ester (50). *N*-Boc-3(*S*)-methyl-L-proline (42.5 mg, 0.185 mmol, 1.0 equiv.) was mixed with **49** (53 mg, 0.185 mmol, 1.0 equiv.), BOP reagent (82 mg, 0.185 mmol, 1.0 equiv.), and Et_3N (28 μL, 0.2035 mmol, 1.1 equiv.) in dry acetonitrile (2.8 mL) and stirred at room temperature under an inert atmosphere for 4 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2 M HCl, water, 10% $NaHCO_3$ (aq.), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by means of flash silica gel column chromatography with 40% EtOAc/Hex to give (**50**) (69.5 mg, 75%) as a colorless glass. ^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.00 (3H, d, J=7.0 Hz), 1.33 (1H, m), 1.38 (9H, s), 1.54 (3H, s), 1.55 (3H, s), 1.72 (1H, m), 1.97 (1H, m), 2.89 (1H, br s), 3.12 (1H, m), 3.18 (1H, dd, J=0, 8.4 Hz), 3.35 (2H, m), 3.45 (3H, s), 3.64 (1H, d, J=5.5 Hz), 4.73 (1H, dd, J=7.3, 14.6 Hz), 5.03 (1H, d, J=10.3 Hz), 5.10 (1H, d, J=17.6 Hz), 6.21 (1H, ddd, J=1.5, 10.6, 17.6 Hz), 6.94 (1H, t, J=7.3 Hz), 7.01 (1H, t, J=7.0 Hz), 7.33 (1H, d, J=8.0 Hz), 7.37 (1H, br s), 7.47 (1H, d, J=8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.4, 27.5, 27.6, 28.1, 29.67, 31.5, 39.1, 45.7, 52.1, 52.9 (d, J_{C-C}=63 Hz), 67.9 (d, J_{C-C}=53 Hz), 80.5, 105.2, 110.4, 112.4, 117.6, 119.9, 121.7, 130.1, 134.0, 140.6, 145.6, 154.6, 172.2, 172.7. IR (neat, NaCl): 3337, 2968, 2929, 2875, 1703, 1625, 1504, 1462, 1435, 1392, 1365, 1248, 1167, 1151, 1120 cm^{-1}. $[\alpha]_D^{25}$=−29.1° (H_2O, c=0.34).

HRMS (FAB+): Calcd for $C_{26}{}^{13}C_2H_{40}N_3O_5$: 500.3035. Found 500.3040 (M+H).

4.7.9. [$^{13}C_2$]-2-(1,1-Dimethyl-2-propenyl)-L-tryptophanyl-3(*S*)-methyl-L-proline (39). Five equivalents of 1N LiOH (230 μL, 0.23 mmol) were added to a 0.03 M THF/H_2O (2:1) solution of **50** (23 mg, 0.046 mmol). The mixture was stirred at room temperature for 24 h. The THF was removed in vacuo and the residue was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting three times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was re-suspended in CH_2Cl_2 (0.5 mL), TFA was added (74 μL, 0.92 mmol, 20 equiv.), and the mixture was stirred for 24 h at room temperature. The CH_2Cl_2 and excess TFA were removed under reduced pressure. The residue was dissolved in water and extracted with EtOAc three times. The aqueous layer was then evaporated to dryness. The residue was re-suspended in water, loaded onto a suitably prepared Dowex ion exchange column, desalted with de-ionized water, and eluted with 2% aqueous NH_4OH. The eluate was evaporated in vacuo and then re-suspended in a small amount of de-ionized water. Finally, the resulting solution was lyophilized to give compound **39** as a white solid (12.3 mg, 0.032 mmol, 69% yield). ^{1}H NMR (400 MHz, D_2O): δ 1.00 (3H, d, *J*=6.2 Hz), 1.46 (1H, m), 1.58 (3H, s), 1.58 (3H, s), 1.88 (2H, m), 2.94 (1H, m), 3.19 (2H, m), 3.35 (1H, m), 3.35 (1H, dd, *J*=5.5, 14.8 Hz), 4.59 (1H, m), 5.20 (1H, dd, *J*=0.8, 10.5 Hz), 5.23 (1H, dd, *J*=0, 17.5 Hz), 6.27 (1H, dd, *J*=10.5, 17.5 Hz), 7.13 (1H, t, *J*=7.0 Hz), 7.19 (1H, t, *J*=7.0 Hz), 7.42 (1H, d, *J*=8.2 Hz), 7.65 (1H, d, *J*=7.8 Hz). ^{13}C NMR (100 MHz, D_2O): δ 17.0, 27.2, 27.3, 32.5, 38.9, 39.0, 45.6, 56.9 (d, J_{C-C}=54 Hz), 66.19 (d, J_{C-C}=52 Hz), 106.1 (d, J_{C-C}=5 Hz), 111.1, 111.8, 118.4, 119.4, 121.4, 129.6, 134.6, 142.0, 146.3, 170.6, 178.4. IR (NaCl, CH_2Cl_2): 3630–1700 (broad), 3292, 3056, 2964, 2964, 2929, 2872, 1558, 1540, 1457, 1362, 921, 743 cm^{-1}. $[\alpha]_D^{25}$=+8.0° (*c*=0.1, H_2O). HRMS (FAB^+) calcd for $C_{20}{}^{13}C_2H_{30}N_3O_3$ 386.2354. Found 386.2359 (M+H).

4.8. Feeding experiments with *P. fellutanum*

Spores from *P. fellutanum* suspended in a 15% aqueous glycerol solution were spread onto malt extract agar slants (20 g malt extract, 20 g glucose, 1 g peptone and 20 g agar per liter of distilled, de-ionized water); 50 μL of the suspension was used per slant. The slants were placed in an incubator at 25°C for 7–10 days. The spores from two slants were scraped into each 4-L flask containing 400 mL of sterile glucose corn steep liquor (40 g glucose and 22 g corn steep liquor per one liter of distilled de-ionized water). The inoculated flasks were placed in an incubator at 25°C for 5–7 days. The glucose corn steep liquor was removed leaving a disk of the fungus. The undersides of the disks, the mycelial cells, were rinsed with 100 mL of sterile water.

Sterile trace element solution (35 mM $NaNO_3$, 5.7 mM K_2HPO_4, 4.2 mM $MgSO_4$, 1.3 mM KCl, 36 μM $FeSO_4{\cdot}7H_2O$, 25 μM $MnSO_4{\cdot}H_2O$, 7 μM $ZnSO_4{\cdot}7H_2O$, 1.5 μM $CuCl_2{\cdot}2H_2O$) containing the biosynthetic precursor (see Table 2 for the volume and molarity of each solution) was placed into each of two flasks containing the fungus. Two control flasks were also set up, containing only sterile trace element solution, for each feeding experiment. The flasks were put into the incubator at 25°C for 10 days and swirled daily to ensure even distribution of the precursor.

A detergent had to be used to dissolve the water-insoluble proposed precursors (**35** and **36**). The water-insoluble compounds were dissolved in a 10% solution of absolute ethanol/chloroform. Tween 80 (0.2 mL) was added to the dissolved precursor. The samples were then sonicated at 40°C under a sterile stream of argon until the solvent was completely removed. Sterile trace element solution was added to the each precursor/Tween 80 residue (see Table 2 for the volume and molarity of each solution) and the mixture was sonicated for ~15 min to aid in micelle formation. Each of the solutions was then placed into two flasks containing the fungus. Two control flasks were also set up, each containing only sterile trace element solution. The flasks were put into the incubator at 25°C for 10 days and swirled daily to ensure even distribution of the precursor.

The aqueous media was decanted off and stored at 4°C with 1–2 mL of chloroform. The mycelial cells from each flask were harvested, combined with the cells from the duplicate experiment and homogenized with 500 mL of methanol in an Oster blender. The methanol suspensions of mycelial cells were placed in a shaker at room temperature for 24 h. Celite (10 g) was added to each suspension before filtering through Whatman #2 paper. The filtrate was stored at 4°C. The residual mycelia and Celite were re-suspended in methanol, placed in the shaker for an additional 42 h and re-filtered.

The methanol solutions from both filtrations were combined and evaporated in vacuo. The aqueous solution from each feeding experiment was added to the corresponding methanolic residue and the mixture was acidified to pH 4 with 12 mL of glacial acetic acid. The acidic solution was extracted four times with 150 mL portions of ethyl acetate. The organic layer was discarded. The aqueous layer was brought to pH 9–10 by the addition of 50 mL of 5 M NaOH. The aqueous layer was then extracted four times with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium carbonate and evaporated to dryness. The paraherquamide from each feeding experiment was purified via radial chromatography and thin layer chromatography with a gradient elution of 4–10% methanol in methylene chloride (Table 3).

4.9. Calculation of the percentage incorporation from ^{13}C NMR data

To compensate for the effect of variable nuclear Overhauser enhancements and different relaxation times that cause carbon signal integrals to differ within the same spectrum, the relative peak heights of labeled and unlabeled paraherquamide A were compared. First, the ratio of the integral of the labeled peak to the added integrals of the rest of the peaks was obtained. Then, the ratio of the integral of the corresponding peak in the unlabeled spectrum to the added integrals in the rest of the spectrum was found. The total percentage of ^{13}C at the labeled position was found by

Table 3. Paraherquamide A isolated from feeding experiments

Proposed precursor	Amt. precursor (mmol)	Molarity (M)	Volume (mL)	Amt. **1** produced (mmol)
21	0.152	7.60×10^{-4}	100	0.0405
22	0.161	8.04×10^{-4}	100	0.0486
24	0.155	7.75×10^{-4}	100	0.0527
27	0.043	4.30×10^{-4}	50	0.0420
Control	–	–	100	0.0222
24	0.157	7.85×10^{-4}	100	0.0283
Control	–	–	100	0.0193
25	0.081	5.45×10^{-4}	75	0.0194
26	0.120	6.00×10^{-4}	100	0.0170
Control	–	–	100	0.0182
25	0.028	7.85×10^{-4}	50	0.0231
26	0.0914	7.85×10^{-4}	75	0.0141
Control	–	–	75	0.0277
23	0.149	7.45×10^{-4}	100	0.0109
Control	–	–	100	0.0182
32	0.169	8.45×10^{-4}	100	0.0405
Control	–	–	100	0.0101
35	0.0424	2.12×10^{-4}	100	0.0284
36	0.0594	2.97×10^{-4}	100	0.0347
Control	–	–	100	0.0217
37	0.126	6.30×10^{-4}	100	0.0263
38	0.040	2.00×10^{-4}	100	0.0103
39	0.031	1.56×10^{-4}	100	0.0146
Control	–	–	100	0.0123

Table 4. Incorporations calculated from electrospray MS data

Compound	mmol	**1** produced (mmol)	Single-label incorporation (%)	Double-label incorporation (%)
21	0.152	0.041	1.2	–
22	0.164	0.049	0.4	–
23	0.149	0.011	0	–
24	0.155	0.053	3.0	–
24	0.157	0. 028	2.2	–
25	0.081	0.019	1.6	0.3
25	0.028	0.023	0.77	0.25
26	0.120	0.017	0.5	0.08
26	0.091	0.014	1.5	0.3
27	0.043	0.042	0.61	0
32	0.169	0.041	10.1	–
35	0.042	0.028	0	0
36	0.059	0.035	0	0
37	0.098	0.026	0	–
38[a]	0.040	0.010	N/A	N/A
39[a]	0.031	0.015	N/A	N/A

[a] After several attempts, we were unable to obtain reliable mass spectral data for the paraherquamide A isolated from the feeding experiments performed with this compound.

dividing the ratio of integrals obtained from the labeled paraherquamide A by the ratio from the unlabeled paraherquamide A and multiplying by 1.1%, the natural abundance of ^{13}C. The percentage of ^{13}C enrichment at the labeled position was obtained by subtracting 1.1% from the total percentage of ^{13}C. The percentage of precursor incorporated was determined by multiplying the percentage of ^{13}C enrichment by the mmol of paraherquamide A produced and then dividing this number by the mmol of isotopically enriched precursor.

4.10. Calculation of percentage incorporation from mass spectra data

Results were calculated by means of electrospray mass spectroscopy, thus the base peak was the M+H peak and was set to 100%. Percentage of [^{13}C] enrichment in paraherquamide A from ^{13}C-labeled biosynthetic precursors was determined according to the method outlined by Lambert et al.[14] The percentage of precursor incorporated was determined by multiplying the percentage of ^{13}C enrichment by the mmol of paraherquamide A produced and then dividing this number by the mmol of isotopically enriched precursor (Table 4).

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