Biosynthesis of polyoxypeptin A: novel amino acid 3-hydroxy-3-methylproline derived from isoleucine

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Polyoxypeptin A is a hexadepsipeptide, isolated from *Streptomyces*, containing the novel amino acid (2*S*,3*R*)-3-hydroxy-3-methylproline. Its biosynthetic pathway is studied by incorporation of stable isotope-labelled amino acids and carboxylic acids. (2*S*,3*R*)-3-Hydroxy-3-methylproline is shown to be derived from isoleucine but not from proline. Isoleucine is also incorporated into the alkyl moiety of the C_{15} acyl side chain, possibly through α-methylbutyryl-CoA. The piperazic acid moieties are synthesized from glutamate. Other hydroxylated amino acids are shown to be derived from each corresponding amino acid.

Introduction

Most anticancer agents are known to induce apoptosis in cultured cells. However, human solid carcinoma cells are often resistant to anticancer drug-induced apoptosis. In the course of our screening for apoptosis inducers, we isolated polyoxypeptin A (Fig. 1) from the culture broth of a *Streptomyces* strain,[1,2] together with other novel products, polyoxypeptin B[2] and chloptosin.[3] Polyoxypeptin A induced apoptosis in apoptosis-resistant human pancreatic adenocarcinoma AsPC-1 cells.[4] The cyclic hexadepsipeptide structure of polyoxypeptin A consists of *N*-hydroxy-L-valine (*N*-OHVal), (2*S*,3*R*)-3-hydroxy-3-methylproline (3-OH,MePro), (3*R*,5*R*)-5-hydroxypiperazic acid (5-OHPip), *N*-hydroxy-L-alanine (*N*-OHAla), (3*R*)-piperazic acid (Pip), and (2*S*,3*S*)-3-hydroxyleucine (3-OHLeu). The peptide was *N*-acylated with a C_{15} side chain. Its stereochemistry was determined by X-ray crystallographic analysis and degradation studies. 3-OH,MePro is a simple but novel amino acid. Therefore, we studied the biosynthesis of polyoxypeptin A by incorporation of stable isotope-labelled amino acids and carboxylic acids.

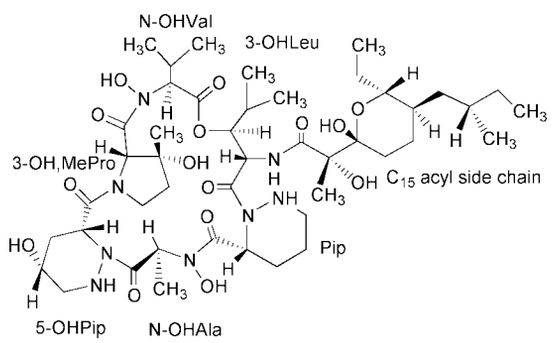

Fig. 1 Polyoxypeptin A.

Results and discussion

Incorporation of ^{13}C-labelled amino acids into polyoxypeptin A is summarized in Table 1. When L-[1,2-$^{13}C_2$]leucine was added to the culture, C-1 and C-2 of the 3-OHLeu were strongly enriched, and a pair of ^{13}C–^{13}C spin couplings was observed. L-[1-^{13}C]Valine and L[1-^{13}C]alanine were also incorporated into their respective amino acids. Interestingly, L-[1-^{13}C]proline was not incorporated into the 3-OH,MePro, but was incorporated into the carbonyl carbons of Pip and 5-OHPip moieties, possibly through conversion to glutamate.[5] DL-[4-^{13}C]proline was not incorporated into any carbon (data not shown). The addition of [Me-^{13}C]methionine showed no significant enhancement of any carbon including the C-Me group of 3-OH,MePro (data not shown). Therefore, 3-OH,MePro is unlikely to be synthesized from proline following its methylation.

Most interestingly, L-[1-^{13}C]isoleucine was efficiently incorporated into the carbonyl carbon of 3-OH,MePro. This synthetic pathway was confirmed by incorporation of L-[^{15}N]isoleucine into N at δ_N 126.9, as shown in Fig. 2. As expected, addition of L-[U-^{13}C]isoleucine showed enrichments of all six carbons in 3-OH,MePro. Unexpectedly, however, L-[U-^{13}C]isoleucine also enriched three carbon sets having ^{13}C–^{13}C spin couplings: CO:C-2:2-Me (from propionyl-CoA), C-7:C-8:8-Me:C-9:C-10 (from α-methylbutyryl-CoA), and C-11:C-12:C-13 (from propionyl-CoA) in the C_{15} acyl side chain. It is known that isoleucine is converted to succinyl-CoA *via* α-methylbutyryl-CoA and propionyl-CoA.[6] Therefore, we concluded that a new

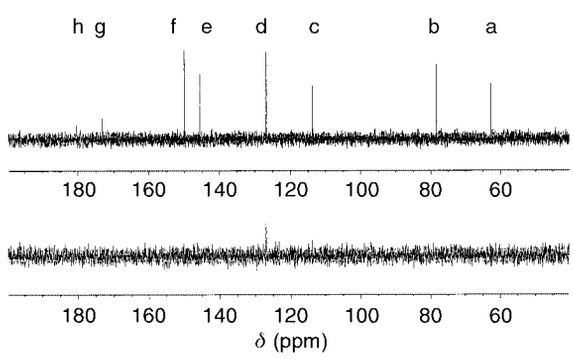

Fig. 2 ^{15}N NMR spectrum of polyoxypeptin A: upper chart, cold polyoxypeptin A, (a) 5-OHPip (N-1) δ 62.8, (b) Pip (N-1) δ 78.5, (c) 3-OHLeu δ 113.8, (d) 3-OH, MePro δ 126.9, (e) 5-OHPip (N-2) δ 145.6, (f) Pip (N-2) δ 150.1, (g) *N*-OHVal δ 180.4 (assigned by ^{15}N–^{1}H HMQC and HMBC); lower chart, enrichment by addition of L-[^{15}N]isoleucine.

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Table 1 Incorporation of ^{13}C-labelled amino acids into polyoxypeptin A

Carbon	δ in $CDCl_3$	Enrichment factor[a]							
		[1,2-^{13}C]Leu	[1-^{13}C]Val	[1-^{13}C]Ala	[1-^{13}C]Pro	[5-^{13}C]Glu	[1,2-^{13}C]Gln	[1-^{13}C]Ile	[U-^{13}C]Ile
N-OHVal CO	169.4	1.4	10.5	1.0	0.9	0.8	0.9	0.9	0.8
2	62.7	1.2	0.8	1.0	1.0	1.0	1.0	1.0	1.0
3	29.4	OL	OL	OL	OL	OL	OL	OL	OL
4	19.4	1.1	0.6	1.0	1.0	1.0	1.0	1.0	1.1
4′	19.7	0.9	0.6	1.0	1.1	1.0	1.0	1.1	1.1
3-OH,MePro CO	166.0	1.0	1.6	0.9	1.2	0.9	1.0	16.6	7.4[b]
2	68.1	1.0	0.7	0.8	0.9	1.0	1.0	0.8	7.9[b]
3	78.4	0.7	0.6	1.0	1.1	1.0	1.0	1.1	7.8[b]
3-Me	27.3	OL	OL	0.8	0.8	0.9	0.8	1.0	8.3[b]
4	37.3	0.9	0.3	0.9	1.0	1.0	0.9	0.9	8.7[b]
5	45.9	1.1	0.6	1.0	0.9	1.0	0.8	1.0	7.9[b]
5-OHPip CO	170.7	1.3	1.1	1.0	1.9	0.9	1.3[b]	1.0	0.8
3	47.7	1.6	0.6	1.0	0.9	1.0	1.2[b]	1.0	1.0
4	29.4	OL	OL	OL	OL	OL	OL	OL	OL
5	59.0	1.4	0.6	1.0	1.1	1.0	0.9	0.8	0.9
6	54.3	2.1	1.0	1.0	1.0	1.4	0.8	0.9	0.9
N-OHAla CO	179.6	1.3	1.3	4.7	1.0	0.9	1.0	0.9	0.9
2	50.9	1.2	0.7	0.9	0.8	0.9	0.9	0.9	0.9
3	14.6	1.0	0.4	0.8	0.9	0.8	0.9	0.9	0.9
Pip CO	168.2	1.1	0.8	0.8	1.9	0.9	1.3[b]	0.9	0.8
3	50.0	1.7	0.5	1.0	0.9	1.0	1.2[b]	0.9	0.9
4	24.9	OL	OL	OL	OL	OL	OL	OL	OL
5	20.7	1.0	0.6	0.9	1.0	0.9	0.9	0.9	0.9
6	46.6	1.2	0.5	1.0	0.8	1.5	0.8	0.8	0.8
3-OHLeu CO	171.5	8.8	1.1	1.0	0.9	0.9	1.0	1.0	0.9
2	55.9	8.8	1.0	1.0	1.0	1.0	0.9	0.9	0.8
3	77.0	OL	OL	OL	OL	OL	OL	OL	OL
4	29.2	1.6	1.0	0.9	1.2	0.9	0.9	1.0	1.0
5	15.6	0.8	0.7	0.9	1.0	0.8	0.9	0.9	0.9
5′	19.9	0.4	0.7	1.0	1.1	1.0	1.0	1.0	1.1
Acyl chain CO	177.4					0.8	0.9	0.8	2.6[b]
2	76.8					OL	OL	OL	OL
2-Me	20.4					1.0	1.0	1.0	3.8[b]
3	99.0					0.9	0.9	0.9	0.9
4	27.8					0.9	0.8	0.9	0.9
5	23.9					0.8	0.8	0.8	0.9
6	35.8					0.9	0.9	0.9	0.9
7	38.1					0.9	0.9	0.9	6.2[b]
8	31.0					OL	OL	OL	OL
8-Me	18.6					1.0	1.0	1.0	6.6[b]
9	31.0					OL	OL	OL	OL
10	11.6					1.0	0.9	0.9	6.1[b]
11	75.8					1.0	0.9	1.0	3.6[b]
12	24.9					OL	OL	OL	OL
13	8.7					1.0	0.9	0.9	3.6[b]

[a] Enrichment factor (peak area of enriched sample/natural abundance peak area) was calculated from spectra run under essentially identical conditions. [b] Value includes intensity of ^{13}C–^{13}C spin-coupling peaks. OL: Signals overlap with others. Significantly enriched signals are shown by underlining.

amino acid, 3-OH,MePro, and the C_5 alkyl group in polyoxypeptin A are derived from the isoleucine precursor, as shown in Scheme 1.

L-[5-^{13}C]Glutamic acid was incorporated into C-6 of the two piperazic acid moieties (Table 1), as was DL-[5-^{13}C]glutamic acid (data not shown). L-[1,2-$^{13}C_2$]Glutamine was also incorporated into the carbonyl group and C-3 of the two piperazic acid moieties. However, glutamate was not incorporated into 3-OH,MePro. On the other hand, L-[3,3,4,4,5,5-$^{2}H_6$]ornithine was not incorporated into the two piperazic acid moieties (data not shown). L-Proline can be easily converted to L-glutamic acid,[5] which would be incorporated into the carbonyl group of Pip and 5-OHPip as described above. These results suggest that the piperazic acid moiety is synthesized from the glutamate precursor, not *via* ornithine as reported by Arroyo *et al.* to occur in monamycin biosynthesis.[7]

Incorporation experiments of ^{13}C-labelled alkanoic acids into polyoxypeptin A are summarized in Table 2. Addition of sodium [1-^{13}C]acetate and sodium [1,2-$^{13}C_2$]acetate showed that the CO:C-3 and C-6:C-5 carbons of the two piperazic acid moieties, and C-3:C-4 and C-5:C-6 carbons of the acyl side chain were derived from acetate. The addition of sodium [1-^{13}C]-propionate enhanced the carbonyl and C-11 carbon signals of the C_{15} acyl side chain. [1-^{13}C]Butyric acid was more weakly incorporated, and its incorporation pattern was similar to those patterns of [1-^{13}C]acetate and [1-^{13}C]propionate. The addition of D-[1-^{13}C]glucose, D-[U-^{13}C]glucose, and [U-^{13}C]glycerol showed random incorporation.

In conclusion, polyoxypeptin A is considered to be synthesized by the *Streptomyces* strain as shown in Fig. 3. The novel amino acid 3-OH,MePro is derived from isoleucine. In monamycin biosynthesis 4-methylproline was suggested to be derived from leucine by incorporation of radioactive leucine.[7] Proline can be synthesized from glutamic acid.[5] Therefore, the 3-OH,MePro skeleton is likely to be formed by a similar alkyl–N closure of isoleucine, as shown in Scheme 1. Unexpectedly the C_5 alkyl group of the C_{15} acyl side chain was also shown to be derived from isoleucine, possibly through α-methylbutyryl CoA (Scheme 1). Isoleucine was also incorporated into the other part of the acyl side chain through propionate (Fig. 3). The two piperazic acids were shown to be derived from glutamic acids, but the level of incorporation was not very

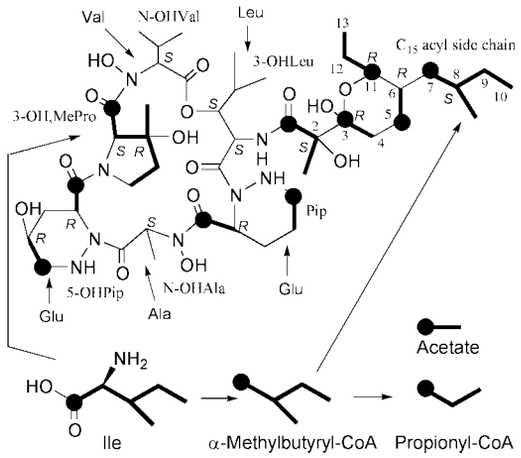
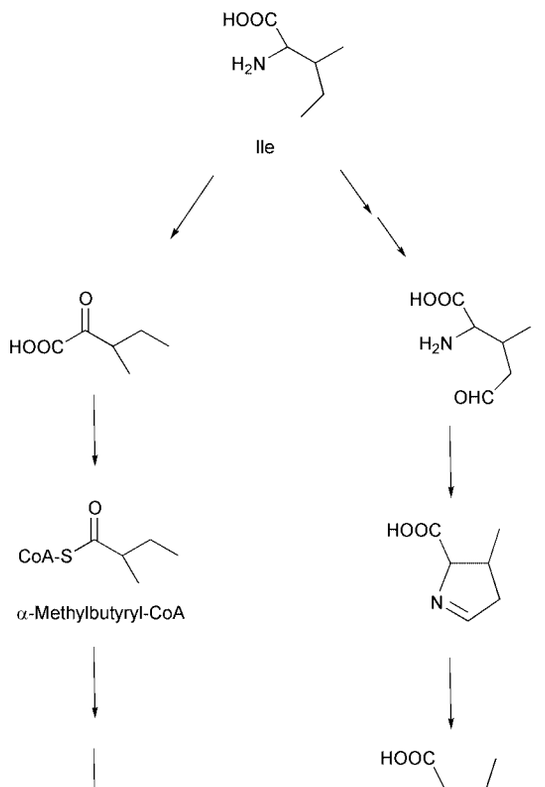


Fig. 3 Incorporation of ^{13}C-labelled amino acids into polyoxypeptin A.



Scheme 1 Proposed pathways for incorporation of isoleucine into 3-hydroxy-3-methylproline and the acyl side chain precursors.

high, and other pathways may be also possible, although the N–N closure of ornithine was clearly excluded. Other hydroxylated amino acids were derived from each corresponding amino acid.

Experimental

Stable isotope-labelled compounds

Most of the stable isotope-labelled compounds were purchased from Sigma or Aldrich, USA. Alkanoic acids and carbohydrates: sodium [1-^{13}C]acetate (99 atom%), sodium [1-^{13}C]-propionate (99%), [1-^{13}C]butyric acid (99%), D-[1-^{13}C]glucose (99%), D-[U-^{13}C]glucose (99%), and [U-^{13}C]glycerol (99%). Amino acids: L-[1,2-$^{13}C_2$]leucine (99%), L-[1-^{13}C]valine (99%), L-[1-^{13}C]alanine (99%), L-[5-^{13}C]glutamic acid (99%), DL-[5-^{13}C]glutamic acid (99%), L-[^{15}N]isoleucine (99%), and L-[Me-^{13}C]methionine (99%). L-[1,2-$^{13}C_2$]Glutamine (99%), L-[1-^{13}C]isoleucine (99%), L-[U-^{13}C]isoleucine (98%), and L-[3,3,4,4,5,5-$^{2}H_6$]ornithine (98%) were purchased from Cambridge Isotope Lab., USA. Sodium [1,2-$^{13}C_2$]acetate (99%), L-[1-^{13}C]proline (99%), and DL-[4-^{13}C]proline (99%) were obtained from Isotec Inc., USA.

Spectral analysis

^{1}H and ^{13}C spectra were measured in $CDCl_3$ with a JEOL JNM-EX400 spectrometer. ^{15}N NMR spectra were taken on a JEOL JMS-A500 spectrometer. ^{1}H NMR spectra were recorded at 400 MHz; and ^{13}C NMR spectra at 100 MHz, using TMS ($\delta = 0$) as internal standard. ^{15}N NMR spectra were recorded at 50.55 MHz using CH_3NO_2 ($\delta_N = 379.6$) as external standard.

Fermentation

Mycelia of *Streptomyces* strain MK498–98F14 were inoculated into a 500-ml baffled Erlenmeyer flask containing a medium (110 ml; pH 7.4) composed of 2.0% D-glucose, 2.0% potato starch (Yoshida Seiyaku), 2.0% glycerol, 1.0% peptone (Polypepton, Wako Pure Chemical), 1.0% meat extract (Kyokuto), 1.0% NaCl, and 0.3% $CaCO_3$. The mycelia were cultured at 30 °C for 3 days on a rotatory shaker at 180 rpm. The seed culture (2.2 ml) was transferred to each of five Erlenmyer flasks containing 110 ml of the same medium as described above, and the mixture was incubated at 28 °C for 6 days on a rotatory shaker at 180 rpm. Each stable isotope-labelled compound was added to 48-hour cultures (amino acid: 5–10 mg, and alkanoic acid: 20 mg in each flask). In the case of addition of isotope-labelled glucose (100–200 mg each), a culture medium composed of 2.0% glycerol, 1.0% soybean meal (Ajinomoto Co.) and 0.5% $CaCO_3$ (pH 7.0) was used. In the case of addition of isotope-labelled glycerol (100 mg each), a culture medium composed of 1.0% glucose, 1.0% potato starch, 1.0% soybean meal and 0.5% $CaCO_3$ (pH 7.0) was used.

Isolation of labelled polyoxypeptin A

The whole culture broth combined from the five flasks (500 ml; pH 7.1–7.8) was extracted with an equal volume of EtOAc, and the extract was evaporated to a syrup. The syrup was purified by column chromatography on silica gel 60 (Kanto Chemical, particle size 40–100 μm; 10–20 g) with EtOAc–hexane (5:3) as the eluent. Fractions were monitored by TLC ($CHCl_3$–MeOH, 20:1) and detected by phosphomolybdic acid–H_2SO_4 reagent consisting of 12 g $NaMoO_4 \cdot 2H_2O$, 7.5 ml 85% H_3PO_3, 25 ml conc. H_2SO_4 and 500 ml H_2O. Fractions containing polyoxypeptin A (R_f 0.65) were collected and concentrated to yield pure labelled polyoxypeptin A (3.8–44.7 mg).

Acknowledgements

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Table 2 Incorporation of ^{13}C-labelled alkanoic acids into polyoxypeptin A

Carbon	δ in $CDCl_3$	Enrichment factor[a]			
		$CH_3{}^{13}CO_2Na$	$^{13}CH_3{}^{13}CO_2Na$	$CH_3CH_2{}^{13}CO_2Na$	$CH_3(CH_2)_2{}^{13}CO_2H$
N-OHVal CO	169.4	1.0	0.9	1.3	0.9
2	62.7	1.0	1.0	1.0	1.0
3	29.4	OL	OL	OL	OL
4	19.4	1.0	1.1	0.9	1.0
4′	19.7	1.0	1.0	0.8	1.0
3-OH,MePro CO	166.0	1.1	1.0	0.9	1.0
2	68.1	0.9	1.0	0.8	1.0
3	78.4	1.0	1.0	1.0	1.0
3-Me	27.3	0.9	0.9	0.9	0.9
4	37.3	1.0	1.0	0.8	1.0
5	45.9	0.9	1.0	1.1	1.0
5-OHPip CO	170.7	1.7	1.6[b]	1.2	1.2
3	47.7	1.0	1.7[b]	1.0	0.9
4	29.4	OL	OL	OL	OL
5	59.0	0.8	2.5[b]	0.8	0.8
6	54.3	2.7	2.4[b]	0.9	1.8
N-OHAla CO	179.6	1.2	1.4[b]	0.9	1.0
2	50.9	0.9	1.2[b]	0.9	0.9
3	14.6	0.9	1.3[b]	1.0	0.9
Pip CO	168.2	1.6	1.8[b]	0.8	1.2
3	50.0	0.9	1.7[b]	1.0	0.9
4	24.9	OL	OL	OL	OL
5	20.7	0.9	3.1[b]	0.7	0.9
6	46.6	2.8	2.4[b]	0.7	1.6
3-OHLeu CO	171.5	1.0	0.9	0.9	0.9
2	55.9	0.9	0.8	0.9	0.8
3	77.0	OL	OL	OL	OL
4	29.2	1.0	0.9	1.2	1.1
5	15.6	0.9	0.8	0.6	0.9
5’	19.9	1.0	1.0	0.8	1.0
Acyl chain CO	177.4	1.1	1.5[b]	6.6	1.3
2	76.8	OL	OL	OL	OL
2-Me	20.4	1.1	1.8[b]	0.9	1.1
3	99.0	3.3	2.0[b]	0.8	1.7
4	27.8	0.9	3.4[b]	0.8	0.8
5	23.9	3.2	3.1[b]	1.0	1.7
6	35.8	0.9	2.8[b]	0.6	0.9
7	38.1	0.9	1.2	0.7	0.9
8	31.0	OL	OL	OL	OL
8-Me	18.6	0.9	1.1	0.8	1.0
9	31.0	OL	OL	OL	OL
10	11.6	1.0	1.0	1.0	1.0
11	75.8	1.1	1.8[b]	8.0	1.6
12	24.9	OL	OL	OL	OL
13	8.7	1.1	1.5[b]	0.9	1.0

[a] Enrichment factor (peak area of enriched sample/natural abundance peak area) was calculated from spectra run under essentially identical conditions. [b] Value includes intensity of ^{13}C–^{13}C spin-coupling peaks. OL: Signals overlap with others. Significantly enriched signals are shown by underlining.

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