Synergy of valine and threonine supplementation on poly(2-hydroxybutyrate-*block*-3-hydroxybutyrate) synthesis in engineered *Escherichia coli* expressing chimeric polyhydroxyalkanoate synthase

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Short title: Val and Thr for P(2HB-*b*-3HB) production

■Abstract

The engineered chimeric polyhydroxyalkanoate (PHA) synthase PhaCAR is composed of

N-terminal portion of *Aeromonas caviae* PHA synthase and C-terminal portion of *Ralstonia eutropha* (*Cupriavidus necator*) PHA synthase. PhaC$_{AR}$ has a unique and useful capacity to synthesize the block PHA copolymer poly(2-hydroxybutyrate-*block*-3-hydroxybutyrate) [P(2HB-*b*-3HB)] in engineered *Escherichia coli* from exogenous 2HB and 3HB. In the present study, we initially attempted to incorporate the amino acid-derived 2-hydroxyalkanoate (2HA) units using PhaC$_{AR}$ and the 2HA-CoA-supplying enzymes lactate dehydrogenase (LdhA) and CoA transferase (HadA). Cells harboring the genes for PhaC$_{AR}$, LdhA, and HadA, as well as for the 3HB-CoA-supplying enzymes β-ketothiolase and acetoacetyl-CoA reductase, were cultivated with supplementation of four hydrophobic amino acids—leucine, valine (Val), isoleucine (Ile), and phenylalanine—in the medium. No hydrophobic amino acid-derived monomers were incorporated into the polymer, which was most likely because of the strict substrate specificity of PhaC$_{AR}$; however, P(2HB-*co*-3HB) was unexpectedly produced with Val supplementation. The copolymer was likely P(2HB-*b*-3HB) based on proton nuclear magnetic resonance analysis. Based on the endogenous pathways in *E. coli*, 2HB units are likely derived from threonine (Thr) through deamination and dihydroxylation. In fact, dual supplementation with Thr and Val showed synergy on the 2HB fraction of the polymer. Val supplementation promoted the 2HB synthesis likely by inhibiting the metabolism of 2-

ketobutyrate into Ile and/or activating Thr dehydratase. In conclusion, the LdhA/HadA/PhaC$_{AR}$ pathway served as the system for the synthesis of P(2HB-*b*-3HB) from biomass-derived carbon sources.

■Introduction

Polyhydroxyalkanoates (PHAs) are bacterial storage polyesters and are used as bio-based and biodegradable plastics (1). Increased interest in these biodegradable plastics in recent years arises from serious petroleum-based plastic pollution that is typically not degrading the marine environment and ecosystem (2). Natural PHAs are composed of (*R*)-3-hydroxyalkanoates (3, 4) and exhibit a variety of properties depending on their monomer composition. The monomer constituents incorporated into PHAs are limited by the substrate specificity of PHA synthase.

PHAs containing 2-hydroxyalkanoate (2HA) units have never been found in nature; therefore, they are considered to be artificial. The first 2HA-polymerizing PHA synthase is an engineered class-II PHA synthase from *Pseudomonas* sp. 61-3 containing two point mutations [PhaC1$_{Ps}$(STQK)] (5), which incorporates lactate (LA) (6), glycolate (7), and 2-hydroxybutyrate (2HB) units (8) into the polymer chain. In addition, PhaC1$_{Ps}$(STQK) incorporates hydrophobic amino acid-derived 2HA units hydroxy-4-methylvalerate

55 (2H4MV), 2-hydroxy-3-methylbutyrate (2H3MB), 2-hydroxy-3-methylvalerate
56 (2H3MV), and 2-hydroxy-3-phenylpropionate (2H3PhP) from leucine (Leu), isoleucine
57 (Ile), valine (Val), and phenylalanine (Phe), respectively (9). The corresponding monomer
58 substrates are supplied through lactate dehydrogenase (LdhA) and CoA transferase
59 (HadA) (Fig. 1) (9). The extremely broad substrate specificity of PhaC1$_{Ps}$(STQK) is
60 useful for synthesizing a variety of artificial PHAs.

61 PhaC$_{AR}$, which is an engineered chimeric enzyme composed of the N-terminal
62 portion of *Aeromonas caviae* PHA synthase (PhaC$_{Ac}$) and C-terminal portion of *Ralstonia*
63 *eutropha* (*Cupriavidus necator*) PHA synthase (PhaC$_{Re}$) (10), is the first class-I enzyme
64 that efficiently incorporates 2HB units (11). Notably, PhaC$_{AR}$ has the unique capacity of
65 synthesizing block PHAs, while PhaC1$_{Ps}$(STQK) and other PHA synthases, in general,
66 synthesize random copolymers (11). PhaC$_{AR}$ spontaneously synthesizes poly(2HB-*block*-
67 3-hydroxybutyrate) [P(2HB-*b*-3HB)] from the mixture of 2HB and 3HB supplemented in
68 the medium. The intact PhaC$_{Re}$ and its derivatives carrying A510X mutations also
69 incorporate 2HB and LA units, respectively, although their capacity for incorporating the
70 2HB units is lower than that of PhaC$_{AR}$ (12, 13).

71 In the present study, we initially attempted to increase the variety of block PHAs
72 synthesized using PhaC$_{AR}$ by introducing other 2HA units into the polymers. We focused

on the LdhA/HadA pathway that supplies the amino acid-derived 2HA monomer substrates (Fig. 1). The 2HA-CoA- and 3HB-CoA-supplying enzymes with $PhaC_{AR}$ were expressed into *Escherichia coli*, and the cells were cultivated together with hydrophobic amino acids. As a result, under those conditions, P(2HB-*b*-3HB) was unexpectedly produced. The pathway served as system for the synthesis of P(2HB-*b*-3HB) from biomass-derived carbon sources.

■Materials and methods

Plasmid construction

pTTQ19ldhAhadA_{Cd_opt} was previously constructed (9). The pBBR1MCS2C_{AR}AB that harbors the genes for PHA synthase (*phaC*$_{AR}$), β-ketothiolase (*phaA*$_{Re}$), and NADPH-dependent acetoacetyl-CoA reductase (*phaB*$_{Re}$) was constructed by first replacing the *Eco*NI site in pBSP$_{Re}$CARpct$_{me}$ (11) with the SpeI site using polymerase chain reaction to yield pBSP$_{Re}$CARpct$_{me}$SpeI. The *Bam*HI/*Afl*II fragment containing partial *pct* was then amplified using pBSP$_{Re}$CARpct$_{me}$ and primers 5′-ATCGGGCCTTTCGAAAGC-3′ and 5′-TTTTTTCTTAAGTTATTTTTTCAGTC-3′, and the *Afl*II/*Spe*I fragment containing *phaAB* was amplified using pTV118NpctC1(STQK)AB and primers 5′-TTTTTTCTTAAGGAAAGGACTACACAATG-3′ and 5′-

TTTTTTACTAGTTCAGCCCATATGC-3′. The *Bam*HI/*Afl*II and *Afl*II/*Spe*I fragments were ligated with the *Bam*HI/*Spe*I-digested $pBSP_{Re}C_{AR}pct_{me}$SpeI to yield $pBSP_{Re}C_{AR}pct_{me}$AB and $pBSP_{Re}C_{AR}pct_{me}$AB was digested with *Eco*RI/*Afl*II, blunted, and self-ligated to eliminate *pct* and yield $pBSP_{Re}C_{AR}$AB. The *Kpn*I/*Xho*I P_{Re} fragment from $pBSP_{Re}C_{AR}$AB was inserted into pBBR1MCS2 (14) at the *Kpn*I and *Xho*I sites to yield $pBBR1M2P_{Re}$. Finally, the *Xho*I/*Spe*I fragments of $pBSP_{Re}C_{AR}$AB containing $phaC_{AR}$, *phaA*, and *phaB* were inserted into $pBBR1M2P_{Re}$ at the *Xho*I and *Spe*I sites to yield $pBBR1MCS2C_{AR}$AB.

Strain and culture conditions

The engineered *E. coli* DH5α was grown on 1.5 mL Luria Bertani medium at 30°C for 12 h as the seed culture. One milliliter or 15 μL of this culture were used to inoculate 100 mL or 1.5 mL M9 of medium (17.1 g $Na_2HPO_4 \cdot 12H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.02 mol $MgSO_4$, and 0.001 mol $CaCl_2$/L distilled water), respectively, containing 1 mM isopropyl β-D-1-thiogalactopyranoside, 20 g/L glucose, and varied concentrations of amino acids and monomer precursors in a 500-mL shake flask or a 10-mL test tube. Under all conditions, 100 mg/L ampicillin and 50 mg/L kanamycin were added to the medium to maintain the plasmids in the cell. The cells were cultivated at 30°C for 72 h,

harvested, and rinsed with distilled water to remove any medium components before lyophilization.

PHA extraction and analysis

PHA was extracted from the lyophilized cells using chloroform at 60°C for 48 h and purified by reprecipitation in an excess amount of hexane and subsequently in methanol. The PHA content and monomer composition were determined using gas chromatography–mass spectrometry (GC–MS, QP2010Plus, Shimadzu) and proton nuclear magnetic resonance (^{1}H NMR) in $CDCl_3$ (400 MHz, JEOL), as previously described (6).

■Results and discussion

Polymer synthesis using PhaC$_{AR}$ with supplementing four hydrophobic amino acids

The engineered *E. coli* expressing PhaC$_{AR}$ and the monomer-supplying enzymes shown in Fig. 1 were cultured in a test tube with amino acid supplementation. None of the amino acid-derived 2HA units (i.e., 2H4MV, 2H3MB, 2H3MV, or 2H3PhP) were incorporated into the polymer (Fig. S1), which differed from the results of PhaC1$_{Ps}$(STQK) (9). Unexpectedly, the 2HB units were incorporated into the polymer with Val

supplementation (Table 1). The decrease in the dry weight of the cell (0.13-fold) after Val supplementation was consistent with that in the results of a previous report (15).

The monomer sequence of the polymer was estimated using ^{1}H NMR (Fig. 2a). The polymer samples for NMR analysis were obtained using the flask-scale cuture (Table S1). The methyne proton of the 2HB units in P(2HB-*co*-3HB) synthesized with Val supplementation exhibited a resonance at 5.0 ppm, which was ascribed to the 2HB–2HB*–2HB triad; therefore, the polymer contained a P(2HB) segment. Similarly, the resonance of the methyne proton of the 3HB units at 5.2 ppm corresponded to a P(3HB) segment. These resonances were identical to those of P(2HB-*b*-3HB) (11) and clearly distinguishable from those of the P(2HB-*ran*-3HB) random copolymer (Fig. 2b). In addition, PhaC$_{AR}$ is not capable of synthesizing P(2HB) homopolymer (11); therefore, the obtained polymer was unlikely to be a homopolymer blend of P(2HB) and P(3HB). Based on these results, the copolymer synthesized with Val supplementation was most likely the block copolymer P(2HB-*b*-3HB).

Generation of the block sequence has been proposed to be the result of changes in intracellular monomer levels during polymer synthesis (11). The enzymatic properties of PhaC$_{AR}$ and the monomer-supplying enzyme propionyl-CoA transferase (PCT) potentially contribute to the characteristic kinetics of polymerization in P(2HB-*b*-3HB)

145 synthesis from exogenous 2HB and 3HB. In the present study, 2HB-CoA and 3HB-CoA
146 were supplied through the LdhA/HadA and PhaA/PhaB pathways, respectively. This
147 result indicated that these monomer suppliers, as well as PCT, can be used for synthesis
148 of block PHA.
149
150 **Polymer production from threonine**
151 The results prompted identification of the 2HB unit precursor. There is no metabolic
152 pathway from Val to 2-ketobutyrate in *E. coli* according to the KEGG pathway
153 (https://www.genome.jp/kegg/pathway.html); therefore, Val was unlikely to be a direct
154 precursor of the 2HB units. To identify the supply route for the 2HB units, the polymer was
155 synthesized using the empty vector pTTQ19. No 2HB units were incorporated using this
156 vector with or without Val supplementation (Table 2), which indicated that LdhA and HadA
157 contributed to the precursor supply route for 2HB. Specifically, 2HB was derived from 2-
158 ketobutyrate. The result suggests that Thr is a 2HB precursor through deamination and
159 dihydroxylation (Fig. 3).
160 To verify the precursor role of Thr, the polymer was synthesized using
161 pTTQ19ldhAhadA$_{Cd_opt}$ with Thr supplementation. For this synthesis, *phaA* and *phaB* were
162 omitted, and 3HB was alternatively supplemented in the medium. The 3HB-CoA supply

163 from exogenous 3HB was less efficient than PhaA and PhaB, and relatively increased the
164 molar fraction of the 2HB units. These conditions facilitated the detection of the 2HB units
165 in the polymer. Expectedly, the 2HB fraction increased with Thr supplementation at all
166 tested concentrations (Table 2), which indicated that they were derived from Thr. The 2HB
167 fraction was detected without Thr supplementation, which should be due to the endogenous
168 Thr. Compared to the result in Table 1 obtained from the test-tube culture, the flask scale
169 culture facilitated the detection of the small amount of 2HB units. The citramalate pathway
170 has also been previously proposed as a 2HB monomer-supplying route (16), although its
171 contribution was not fully verified because of the lack of a proper negative control.
172 We then questioned why Val supplementation increased the accumulation of 2HB units.
173 To verify the role of Val, the dual supplementation of Thr and a small concentration of
174 Val in the medium was tested. The 2HB content and fraction increased with an increase
175 in the Val concentration (Table 3). The result indicated that Val enhanced the supply of
176 2HB derived from Thr. Polymer production decreased with Val supplementation,
177 especially at levels >0.1 g/L, because of the growth inhibition by Val supplementation.
178 Val reportedly inhibits *E. coli* IlvH involved in the biosynthesis of Ile from 2-
179 ketobutyrate (17, 18); therefore, Val supplementation potentially reduces the flux from 2-
180 ketobutyrate to Ile and subsequently increases the flux toward 2HB. In addition, it has

been reported that Val allosterically activates Thr deaminase, which catalyzes the formation of 2-ketobutyrate from Thr (19); therefore, the role of Val should be that of a regulator of amino acid metabolism and not that of a monomer precursor (Fig. 3).

Polymer synthesis with 2HHx, 2H4MV, or 2H3PhP supplementation

$PhaC_{AR}$ incorporated no Leu-, Val-, Ile-, and Phe-derived 2HA units into the polymer (Table 1) most likely because of strict substrate specificity of the enzyme. Substrate specificity of $PhaC_{AR}$ was investigated using 2-hydroxyhexanote (2HHx), which is a structural isomer of 2H4MV, with 2H3PhP tested for comparison. As a result, a trace amount of 2HHx was incorporated into the polymer (Table S2). In contrast, 2H4MV and 2H3PhP units were not detected in the polymer, which indicated that the capacity of $PhaC_{AR}$ to incorporate medium-chain-length (mcl) 2HA-CoA is considerably lower than that of $PhaC1_{Ps}$(STQK).

In conclusion, P(2HB-*co*-3HB) was synthesized in *E. coli* using the LdhA/HadA pathway that supplied 2HB-CoA from Thr (Fig. 1). The copolymer was most likely P(2HB-*b*-3HB) based on the ^{1}H NMR analysis and previous finding that $PhaC_{AR}$ does not synthesize P(2HB) hompolymer; therefore, this is the first report of P(2HB-*b*-3HB)

199 synthesis from biomass-derived carbon sources. Dual supplementation with Thr and Val
200 synergistically increased the 2HB fraction in the polymer. Val likely promoted the
201 conversion of Thr into 2-ketobutyrate through deamination and dihydroxylation. $PhaC_{AR}$
202 did not recognize mcl-2HA monomers with the exception of incorporating a trace amount
203 of 2HHx units. The strict substrate specificity of $PhaC_{AR}$ was beneficial for synthesizing
204 the block PHA with the finely controlled monomer composition compared to synthesis
205 using $PhaC1_{Ps}$(STQK), which exhibits broad substrate specificity and synthesizes
206 multicomponent copolymers.
207
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278 Figure captions

279 FIG. 1. Poly(2-hydroxyalkanoate-*co*-3-hydroxybutyrate) [P(2HA-*co*-3HB)] biosynthesis
280 pathways in engineered *Escherichia coli* with amino acid supplementation. Amino acids
281 are also supplied through the endogenous pathways.

282

283 FIG. 2. ^{1}H NMR analysis of the copolymer poly(8.3 mol% 2-hydroxybutyrate-*co*-3-
284 hydroxybutyrate) [P(2HB-*co*-3HB)] synthesized with Val supplementation in engineered
285 *Escherichia coli* harboring pBBR1MCS2C$_{AR}$AB and pTTQldhAhadA$_{Cd_opt}$ (A) and
286 P(2HB-*random*-3HB) (B). The resonances at 4.8–5.1 ppm are ascribed to the methyne
287 proton of the 2HB units in the following four triad sequences: 2HB–2HB*–2HB (i), 2HB–
288 2HB*–3HB and/or 3HB–2HB*–2HB (ii, iii), and 3HB–2HB*–3HB (iv). The resonance
289 at 5.2–5.4 ppm is ascribed to the methyne proton of the 3HB units in the dyad sequences
290 of 3HB–3HB* and 2HB–3HB*.

291

292 FIG. 3. Poly(2-hydroxybutyrate-*block*-3-hydroxybutyrate) [P(2HA-*b*-3HB)] biosynthesis
293 pathways from threonine in engineered *Escherichia coli* and proposed role of valine.
294 Amino acids are also supplied through the endogenous pathways.

295

Table 1. Polymer production with supplementation of four hydrophobic amino acids[a]

Amino acids	CDW (g/L)	Polymer production (g/L)	Monomer composition(mol%)	
			2HB	3HB
-	4.00 ± 0.68	0.52 ± 0.06	N.D.	100
Leu	5.47 ± 0.09	0.61 ± 0.01	N.D.	100
Val	0.51 ± 0.11	0.38 ± 0.01	3.6	96.4
Ile	3.73 ± 0.52	0.45 ± 0.49	N.D.	100
Phe	5.04 ± 0.31	0.49 ± 0.02	N.D.	100

[a]Polymers were synthesized in recombinant *Escherichia coli* harboring pBBR1MCS2C$_{AR}$AB and pTTQ19ldhAhadA$_{Cd_opt}$ with amino acid supplementation (1 g/L each) in a test tube. CDW, cell dry weight; HB, hydroxybutyrate; N.D., not detected. Data are means ± standard deviations of three independent experiments. Monomer composition was determined by gas chromatography–mass spectrometry (GC–MS).

Table 2. Polymer production in *Escherichia coli* with supplementation of valine (Val) and threonine (Thr)[a]

Plasmids	Val conc. (g/L)	Thr conc. (g/L)	CDW (g/L)	Polymer production (g/L)	Monomer composition (mol%) [b]	
					2HB	3HB
pTTQ19 (empty vector), pBBR1MCS2C$_{AR}$AB	0	0	3.59	1.51	0	100
	1	0	0.64	0.37	0	100
pTTQ19ldhAhadA$_{Cd_opt}$, pBBR1MCS2C$_{AR}$ [c]	0	0	0.60	0.16	4.6	95.4
	0	1	0.92	0.18	6.7	93.3
	0	3	0.68	0.16	5.5	94.5
	0	10	0.77	0.23	8.7	91.3
	0	15	0.64	0.20	6.8	93.2

[a]Cells were cultured in a shake flask. [b]Monomer composition was determined by ^{1}H NMR. HB, hydroxybutyrate. [c]Cells were cultivated with 0.5 g/L (*R,S*)-3HB-Na.

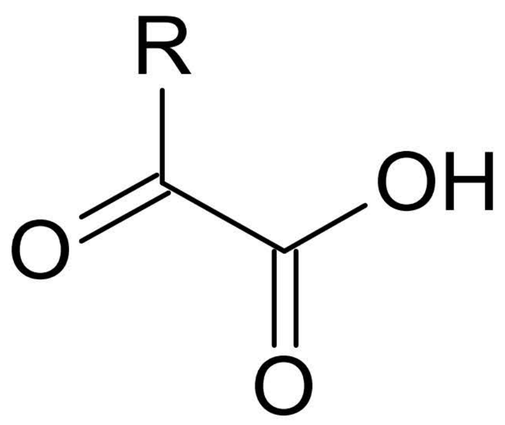
Table 3. Polymer production in *Escherichia coli* with dual supplementation of valine (Val) and threonine (Thr) [a]

Val conc. (g/L)	Thr conc. (g/L)	CDW (g/L)	Polymer production (g/L)	Polymer content (wt%)	2HB content (wt%)	Monomer composition (mol%) [b]	
						2HB	3HB
0	0	2.94 ± 0.28	1.56 ± 0.07	53.7 ± 6.3	0.61	1.0	99.0
0	5	6.45 ± 1.08	3.57 ± 0.73	55.6 ± 7.4	0.81	1.4	98.6
0.001		7.11 ± 0.44	4.70 ± 0.29	66.7 ± 8.3	0.79	1.4	98.6
0.01		4.35 ± 1.82	2.79 ± 1.33	62.2 ± 4.9	1.31	1.9	98.1
0.1		0.56 ± 0.03	0.21 ± 0.01	37.6 ± 1.1	4.20	11.1	88.9
0.5		0.38 ± 0.04	0.13 ± 0.01	35.6 ± 2.4	7.21	18.6	81.4

[a]Polymers were synthesized using *E. coli* harboring pBBR1MCS2C_{AR}AB and pTTQ19ldhAhadA_{Cd_opt} in a shake flask. [b]HB, hydroxybutyrate; 2HB content and monomer composition in the cells were determined by ^{1}H NMR. Data are the means ± standard deviations of three independent experiments. ^{1}H NMR was measured using a single sample.

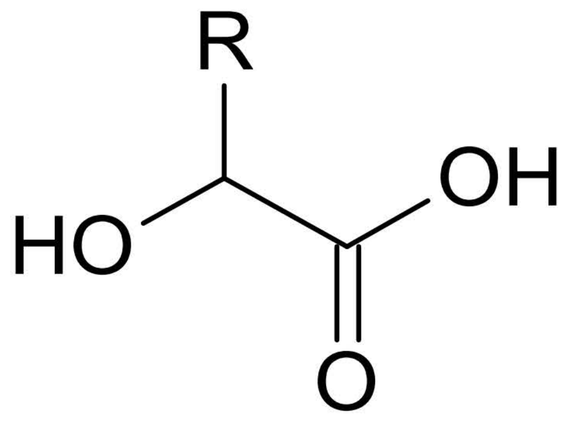
hydrophobic amino acids

glucose



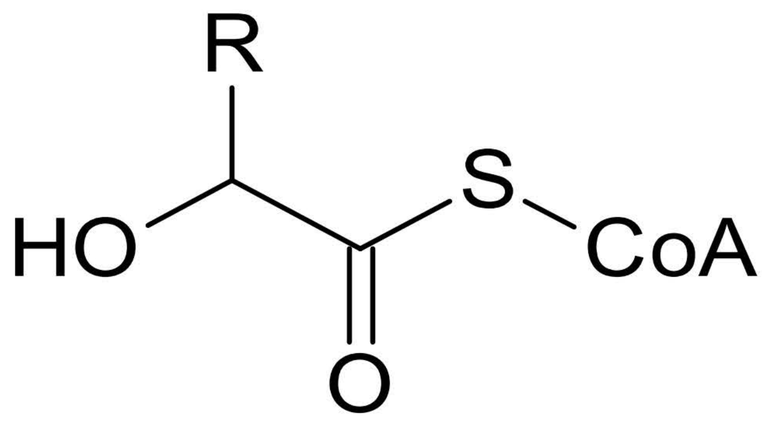
2-ketoalkanoate

LdhA

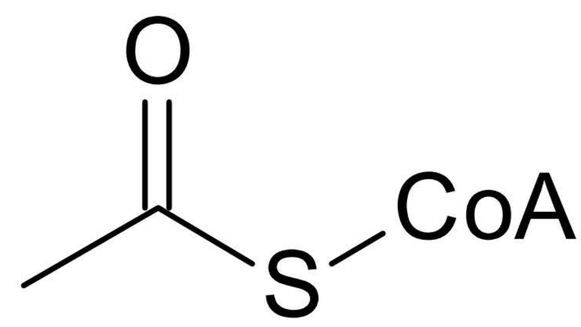


2-hydroxyalkanoate (2HA)

HadA

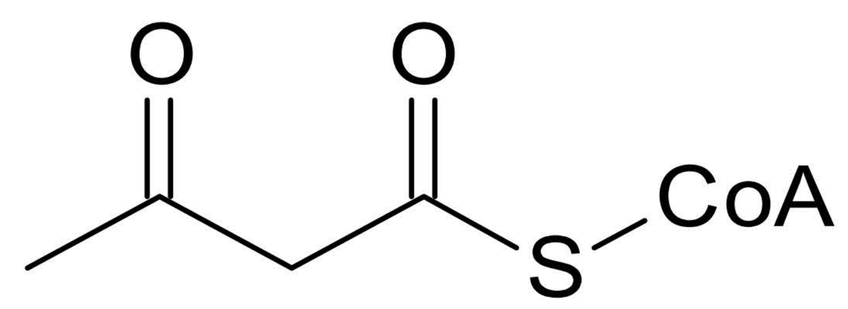


2HA-CoA



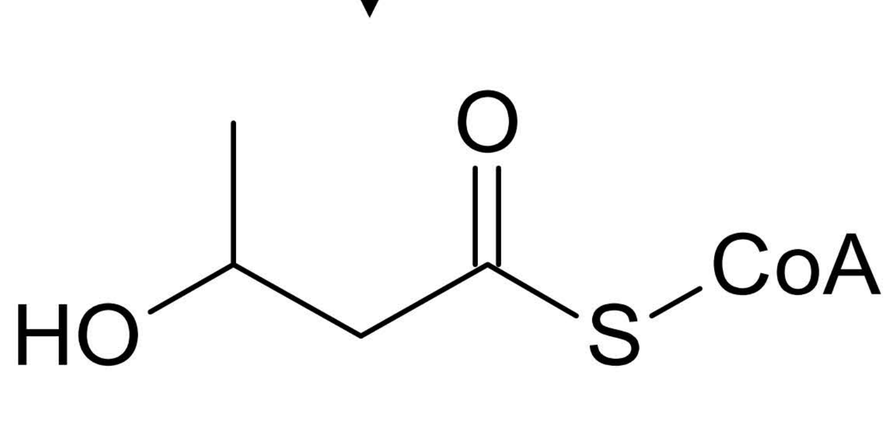
acetyl-CoA

PhaA



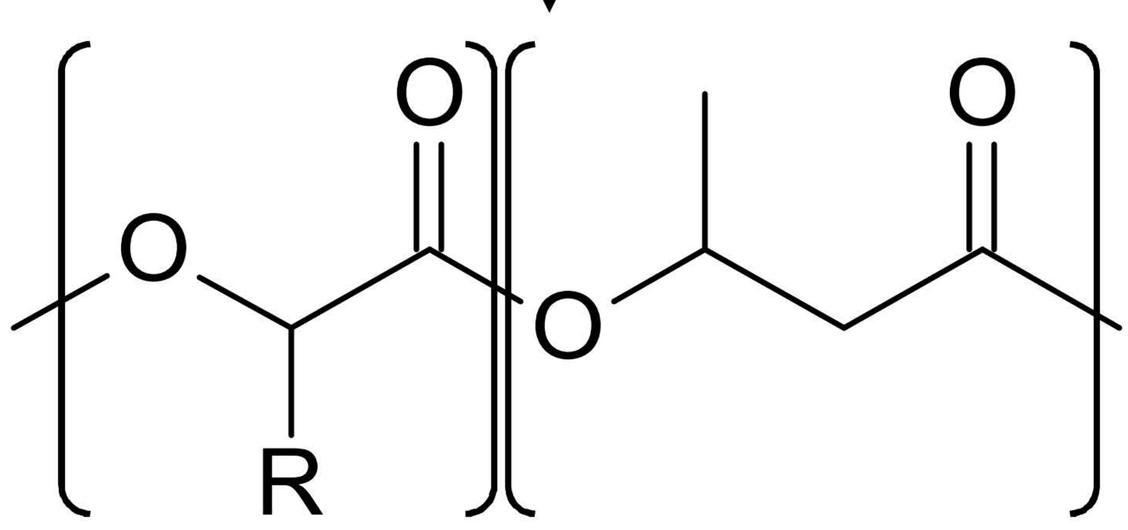
acetoacetyl-CoA

PhaB



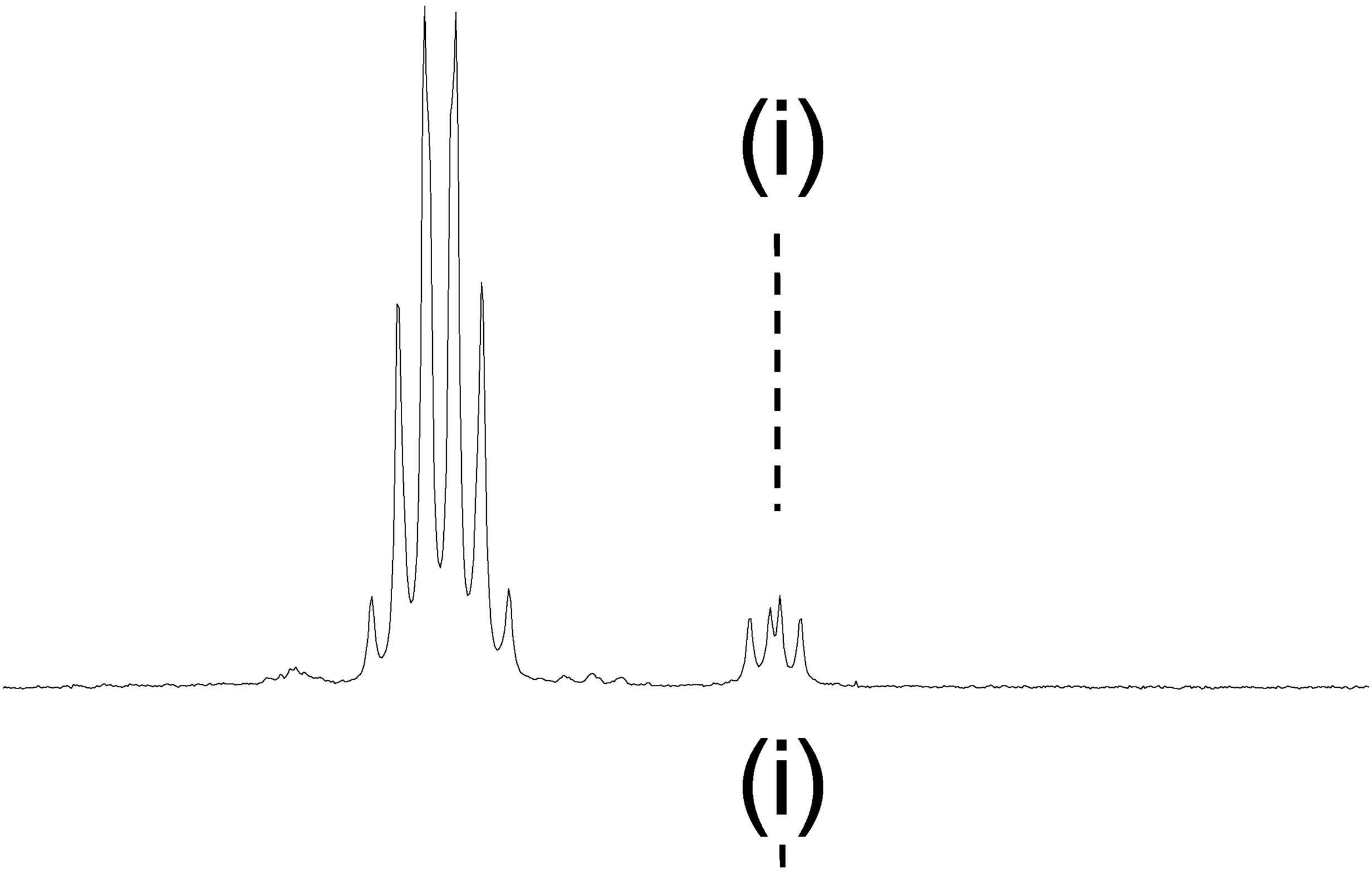
3HB-CoA

PHA synthase

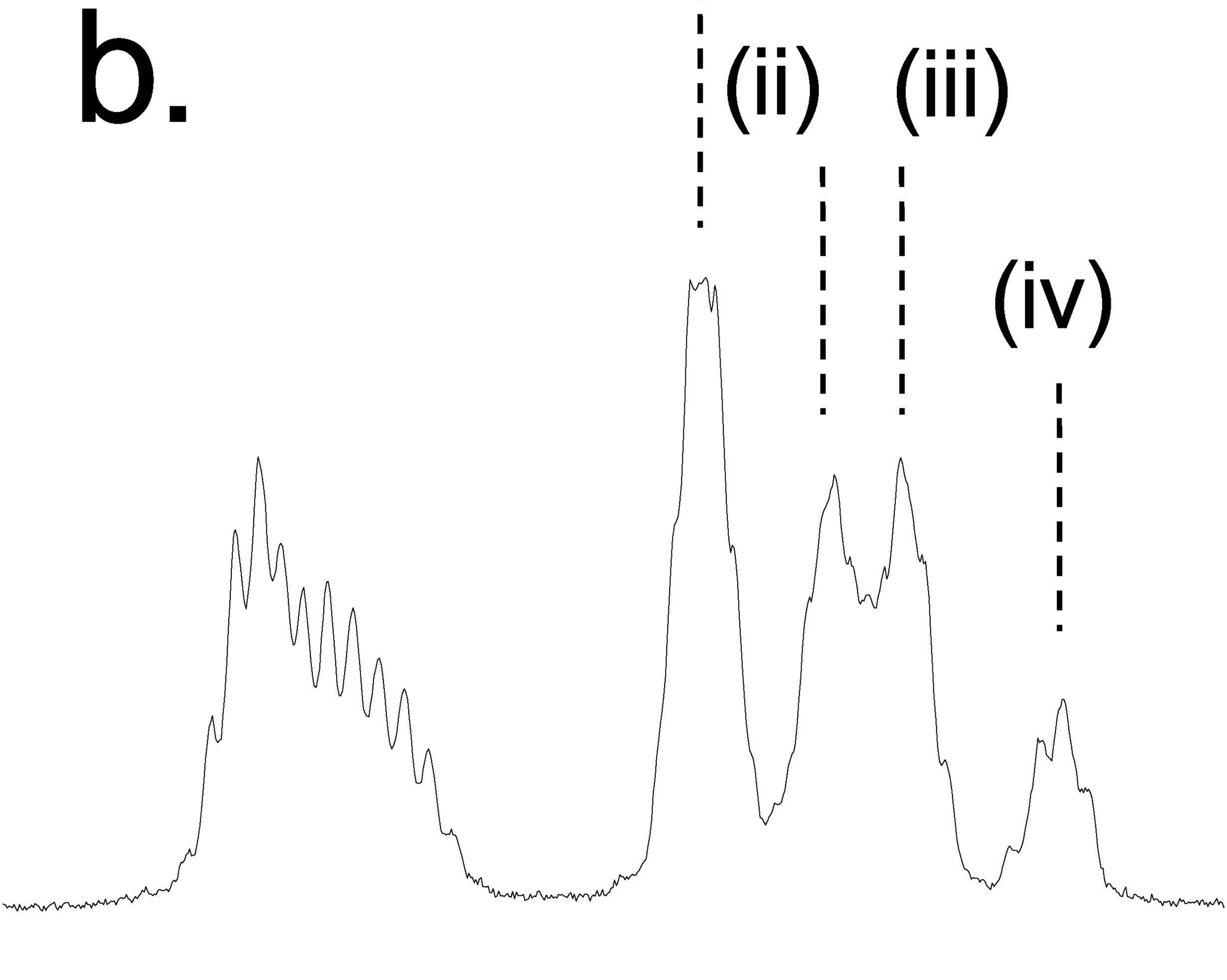


P(2HA-*co*-3HB)

a.



b.

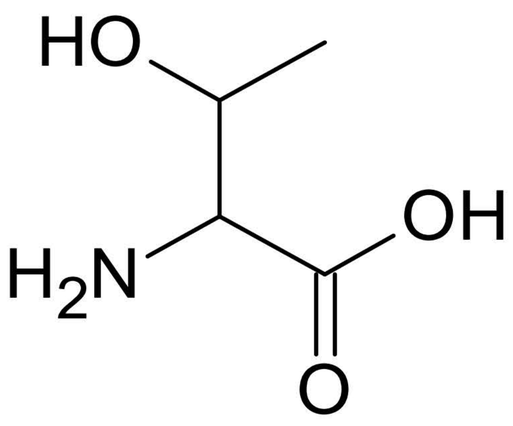


5.4 5.1 4.8
(ppm)

threonine

valine

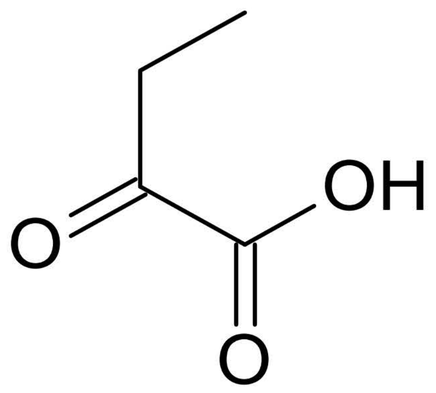
glucose



threonine

+

valine

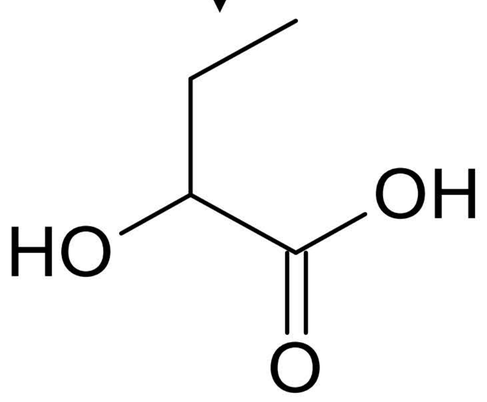


2-ketobutyrate

IlvBH

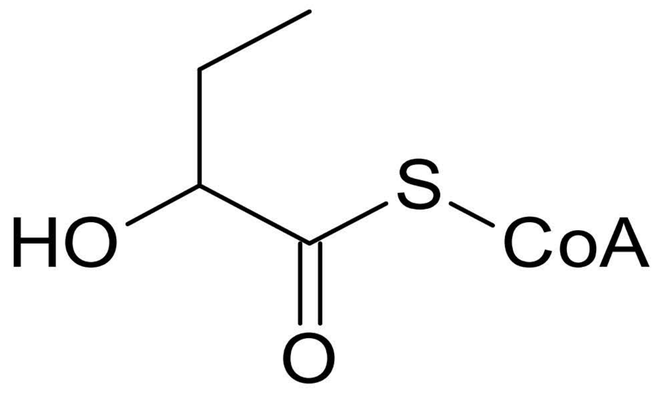
isoleucine

LdhA



2-hydroxybutyrate
(2HB)

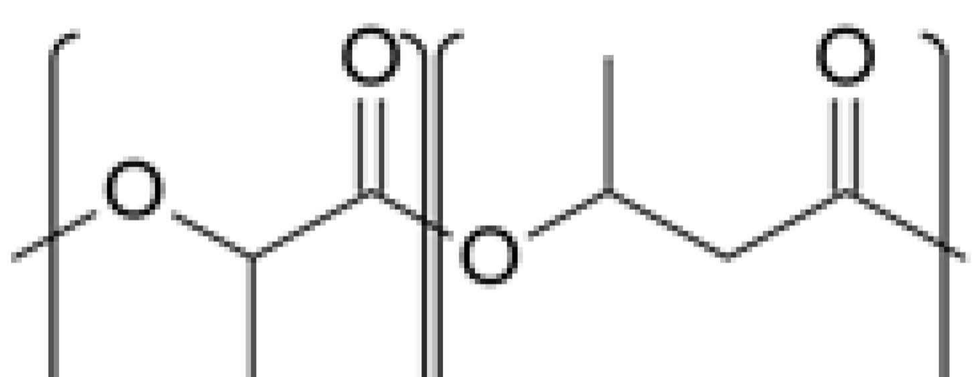
HadA



2HB-CoA

3HB-CoA

$PhaC_{AR}$



P(2HB-*b*-3HB)