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MINI-REVIEW

Biosynthesis and metabolic pathways of pivalic acid

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Abstract Occurrence, biosynthesis, and biodegradation of pivalic acid and other compounds, having a quaternary carbon atom by different bacteria, are described. We have summarized the relevant data that have so far been published, presenting them in a graphical form, i.e., as biodegradation pathways including B_{12}-dependent isomerization and desaturation that lead to the degradation of pivalic acid and similar compounds to products with other than quaternary carbon atoms, i.e., compounds whose catabolism is well known.

Keywords Pivalic acid · Isooctane · Biosynthesis · Biodegradation

Introduction and occurrence in nature

Interest in branched fatty acids and organic compounds, in general, dates back several decades (Kaneda 1991). As noted in a recent review (Dembitsky 2006), hundreds of organic compounds found in nature have in their molecules *tert*-butyl group (*t*-butyl). More than 100 of natural compounds with *t*-butyl group have so far been described (Reaxys 2012), which are biosynthesized by living organisms. The variability of natural compounds with *t*-butyl group can be illustrated (Fig. 1) by, e.g., pivalic acid or the nonproteinogenic amino acid *tert*-leucine (Tle). This amino acid was identified, for instance, in teleocidin derivatives, i.e., in blastmycetin E which is produced by the actinomycete *Streptoverticillium blastmyceticum* (Irie et al. 1990) or in bottromycin A, an antibiotic produced by *Streptomyces bottropensis* whose structure has been revised at least three times (Gouda et al. 2012). A considerable amount of the pivalic acid, which is found in nature, is assumed to be of anthropogenic origin, e.g., arriving from prodrugs (Brass 2002).

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Biosynthesis

Biosynthesis of the *t*-butyl group and, thus, of pivalic acid has not yet been investigated in detail, in contrast to other branched short-chain fatty acids (Kaneda 1991). It is assumed that, in higher multicellular organisms, the *t*-butyl group, e.g., in ginkgolides, is formed by the cleavage of the C–C bond adjacent to a gem-dimethyl unit followed by methylation elicited by *S*-adenosylmethionine (Strømgaard and Nakanishi 2004). A similar mechanism is assumed to be responsible for the appearance of *t*-butyl group in the biosynthesis of the side chain of some sterols (Giner 1993). In bacteria, one of the possible biosynthetic pathways may proceed analogously with the frequently described biosynthesis of branched amino acids, i.e., Val, Leu, and Ile. As noted by Bisel et al. (2008), "the possibility of *t*-valeryl (picolinyl) starter unit derived from Tle is very attractive for the unusual terminal *t*-butyl group in many compounds containing this unusual group," but the hypothesis lacks experimental support.

Pivalic acid, i.e., 2,2-dimethylpropionic acid, is found in nature free or in the form of derivatives or homologues

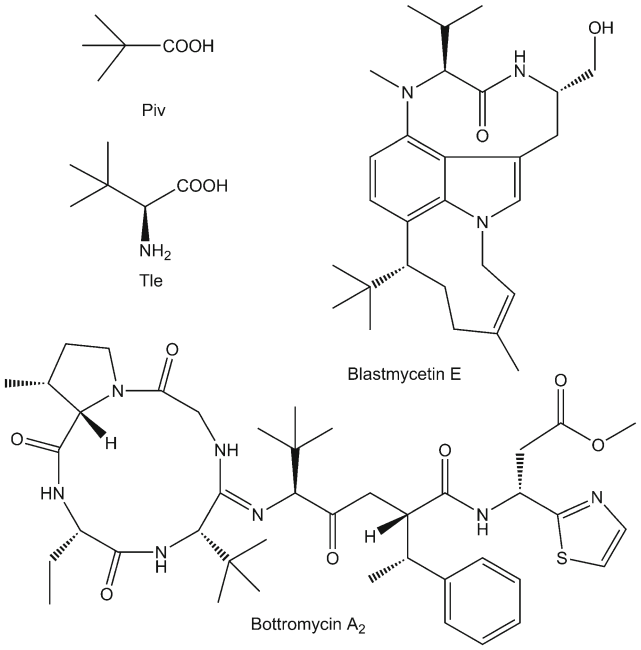

Fig. 1 Natural compounds with *t*-butyl group

(Dembitsky 2006). Its biosynthesis has not been fully elucidated. A recent study (Cracan and Banerjee 2012) has included experiments aiming at elucidating, at least partly, the biosynthesis of pivalic acid. The enzymes *Geobacillus kaustophilus* isobutyryl-CoA mutase fused (*Gk*IcmF), where IcmF is an ICM with ICM and GTPase activities, and *Cm*IcmF (Cm is *Cupriavidus metallidurans*) were used to document the conversion of isovaleryl-CoA to pivalyl-CoA. However, it should be noted that the activity of the enzymes in the isomerization of isovaleryl-CoA to pivaloyl-CoA is two to three orders of magnitude lower than that in the reaction of butyryl-CoA yielding isobutyryl-CoA.

Figure 2 shows the amino acid sequences of selected parts of mutases from different bacteria. They show considerable similarity; to our mind, it is just a matter of time when some bacteria, probably those living in extreme habitats, will be found to possess a mutase with high activity to isovaleryl-CoA, which is similar to the activity of isobutyryl-CoA or methylmalonyl-CoA mutases to isobutyryl- or methylmalonyl-CoA, respectively.

The incorporation of short-chain branched fatty acids (FAs) as starter units was first published in 1971 (Willecke and Pardee 1971). A mutant strain of *Bacillus subtilis* defective in branched chain α-keto acid dehydrogenase was used to incorporate, among others, pivalic acid into fatty acids. Addition of pivalic acid into the medium yielded two homologues, i.e., 12,12-dimethyltridecanoic and 14,14-dimethylpentadecanoic acids in amounts of 13 and 24 % of total FAs, respectively. These data confirmed that even a highly branched acid, such as pivalic acid, can serve as a starter unit in the biosynthesis of FA.

Rezanka et al. (2011) used perdeuterated pivalic acid as a starter unit; three bacteria (*Alicyclobacillus acidoterrestris*, *Rhodococcus erythropolis*, and *Streptomyces avermitilis*) were found to incorporate this acid into FAs, giving rise to *tert*-butyl fatty acids. In *R. erythropolis*, pivalic acid was transformed to two starter units: isobutyric and 2-methylbutyric acids. Both these precursors were employed in the biosynthesis of iso-FAs and even anteiso-FAs. *S. avermitilis* incorporated both pivalic and 2-methylbutyric acids into the antibiotic avermectin. The transformation of pivalic acid to isobutyric and 2-methylbutyric acids opens a potential way to biodegradation of exogenous pivalic acid, arriving from propharmaceuticals or from gasoline additives, such as isooctane. The incorporation of pivalate and the biosynthesis of appropriate fatty acids in bacteria may thus be much more widespread than currently assumed.

Biodegradation

As seen from the schemes below, the biodegradation of compounds, having quaternary carbon atom, poses problems (Sin and Chua 2000). Several strains capable of degrading compounds having quaternary carbon atom (Solano-Serena et al. 2000; Müller et al. 2008; Schäfer et al. 2011) have already been isolated, a pioneering study being performed in 2003 (Probian et al. 2003).

Though pivalic acid was formerly assumed to be completely resistant to bacterial degradation and was used as an internal standard in the determination of volatile FA by rumen microorganisms (Czerkawski 1976), several degradation pathways have recently been proposed (Probian et al. 2003; Solano-Serena et al. 2004). To overcome the problems in the biodegradation, i.e., the removal of the carboxyl group bound to the quaternary carbon, it is necessary to introduce a polar group, e.g., hydroxyl, into the β-position, see Fig. 3. Although the hydroxylation of a methyl group (all three methyl groups are equivalent) is basically an oxidation reaction, it was found to take place (Probian et al. 2003) even in an anoxic environment, i.e., in an environment supporting the growth of denitrifying bacteria. A new pathway, in which nitrous oxide was converted to nitrogen and oxygen that was then used to oxidize methane via methanol to carbon dioxide, was discovered in a special case, viz., in a bacterium named "*Candidatus Methylomirabilis oxifera*" (Ettwig et al. 2010).

One of the methyl groups is thus oxidized to yield dimethylmalonate, which then splits off CO_2 to give isobutyrate. This compound is then metabolized in well-known and frequently described reactions (Probian et al. 2003; Kniemeyer et al. 1999).

A. tertiaricarbonis	70	P F T R G P Y P T M Y R S R T W T M R Q I A G F G T G E D T N … I L K E Y M A Q K E Y I	212
G. kaustophilus	570	P Y T A G V F P F K R Q G - E D P K R Q F A G E G T P E R T N … I L K E D Q G Q N T C I	737
M. algicola	73	P F T R G P Y P T M Y R G R N W T M R Q I A G F G T A R E T N … I L K E Y I A Q K E W I	215
M. alhagi	71	P F T R G P Y P T M Y R G R N W T M R Q I A G F G T G E D T N … I L K E F M A Q K E Y I	214
M. petroleiphilum	69	P F T R G P Y P T M Y R S R T W T M R Q I A G F G T G E D T N … I L K E Y M A Q K E Y I	213
Nocardioides sp.	76	P F T R G P Y P T M Y R G R H W T M R Q I A G F G Q A E E T N … I L K E Y V A Q K E W I	219
P. freudenreichii	69	P F V H G P Y A T M Y A F R P W T I R Q Y A G F S T A K E S N … I L K E F M V R N T Y I	211
R. sphaeroides	71	P F T R G P Y P T M Y R G R N W T M R Q I A G F G T G E D T N … I L K E Y M A Q K E Y I	211
R. sphaeroides		- - - - - - - - - M T Q K D S P W L F R T Y A G H S T A K A S N … L M K E Y L S R G T Y I	135
S. novella	76	P F T R G P Y P T M Y R S R T W T M R Q I A G F G T G E D T N … I L K E Y M A Q K E Y V	218
S. cinnamonensis	59	P F T R G L Y A T G Y R G R T W T I R Q F A G F G N A E Q T N … I F K E Y I A Q K E W L	202
X. autotrophicus	70	P F T R G P Y P T M Y R S R N W T M R Q I A G F G T G E D T N … I L K E Y M A Q K E Y I	212

Fig. 2 Similarity of sequences of different mutases from different bacteria, which catalyze 1,2-rearrangement of some carboxylic acids. *Aquincola tertiaricarbonis* HcmA_L108/PM1, *G. kaustophilus* IcmF_HTA426, *Marinobacter algicola* HcmA_DG893, *Mesorhizobium alhagi* HcmA_CCNWXJ12-2, *Methylibium petroleiphilum* MCM_Mpe_B0541, *Nocardioides* sp. HcmA_JS614, *Propionibacterium freudenreichii* MCM_CIRM-BIA1, *Rhodobacter sphaeroides* HcmA_KD131/17029, *R. sphaeroides* ECM_17029, *Starkeya novella* HcmA_DSM506, *S. cinnamonensis* IcmA_A3823.5, *Xanthobacter autotrophicus* HcmA_Py2. *Blue-typed* amino acids show conserved residues directly involved in substrate binding and interact specifically with the CoA moiety (Ratnatilleke et al. 1999). *Red-typed* amino acids stand for two residues thus far identified to interact specifically with the acyl group of the substrates

Radical intermediates are used in the 1,2-rearrangement of the CoA thioester moiety of CoA-activated carbonic acids, which is catalyzed by cobalamin-dependent CoA-carbonyl mutases. Two mutases have so far been identified in bacteria: methylmalonyl-CoA mutase (EC 5.4.99.2) and isobutyryl-CoA mutase (EC 5.4.99.13). The former uses (*R*)-methylmalonyl-CoA as substrate, which is reversibly and stereospecifically converted to succinyl-CoA. The latter catalyzes the reversible conversion of isobutyryl-CoA into butyryl-CoA. This mutase is less stereospecific than methylmalonyl-CoA mutase (Moore et al. 1995).

Both mutase enzymes have a very narrow spectrum of substrates which they transform, e.g., methylsuccinyl-CoA and ethylmalonyl-CoA (Rétey et al. 1978; Shinichi et al. 1994). The possibility of converting branched acids, i.e., methylmalonate and isobutyrate, into their corresponding straight chain acids by 1,2-rearrangement has spurred the search for other substrates, among others, also pivalic acid.

Another possibility of degradation of pivalic acid consists basically in a "reversal" of its biosynthesis (Cracan and Banerjee 2012). Mutase-catalyzed (EC 5.4.99.-) conversion of pivalyl-CoA leads to 3-methylbutyryl-CoA (Fig. 3), which is, in principle, a starter unit for the biosynthesis of iso-fatty acids. This starter unit arises also catabolically in the sequence Leu→α-keto acid (4-methyl-2-oxo-pentanoic acid)→3-methylbutyric acid. This 1,2-

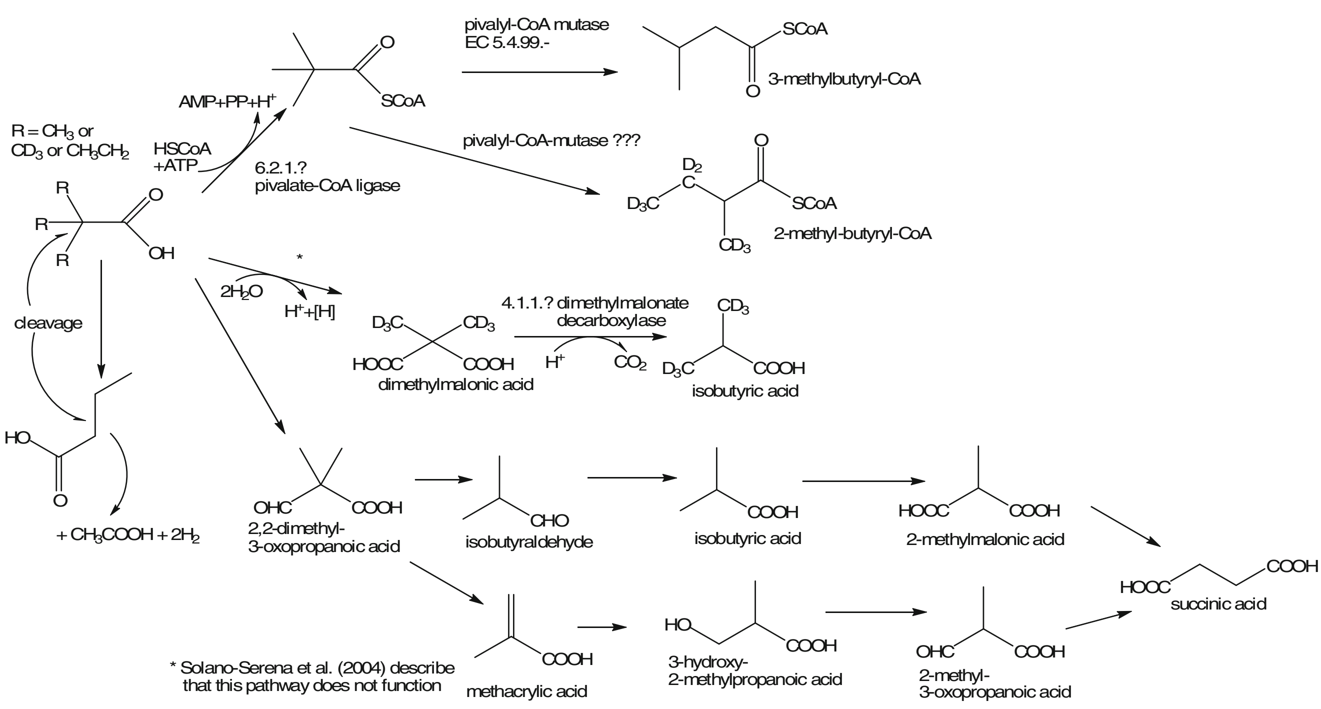

Fig. 3 Biodegradation of pivalic acid

rearrangement could lead to the formation of less branched compounds, i.e., to the formation of tertiary carbon from quaternary carbon.

A third possibility, as shown in our study (Rezanka et al. 2011) (Fig. 3), is a "rearrangement" of pivalic acid to 2-methylbutyryl acid (the rearrangement involves methyl group, not a carboxyl group like in the case of the enzyme EC 5.4.99.-) and its incorporation into anteiso-FA; this has been demonstrated by GC-MS.

Branched aliphatic alkanes with quaternary substituted carbon atoms are known and have been identified in sediments more than one billion years old (Kenig et al. 2003). It is thus clear that they are not of anthropogenic origin and have been produced either abiotically, e.g., by geological processes at high pressures and temperatures, or were biosynthesized by living organisms.

Oil is also rich in hydrocarbons of a similar type, among which isooctane (2,2,4-trimethyl-pentane) is highly regarded in the car industry. Degradation of isooctane, see Fig. 4, proceeds to pivalic acid via hydroxylation of one of the methyl groups of the isopropyl group, oxidation to 2,4,4-trimethylpentanoic acid, and β-oxidation with a simultaneous splitting of propionic acid and formation of the key intermediate, i.e., pivalic acid. Solano-Serena et al. (2004) further suggest a hypothetical degradation of pivalic acid that has, as yet, not been experimentally confirmed.

Conclusion

In conclusion, several facts should be stressed. To our knowledge, until this moment (June 2012), three different

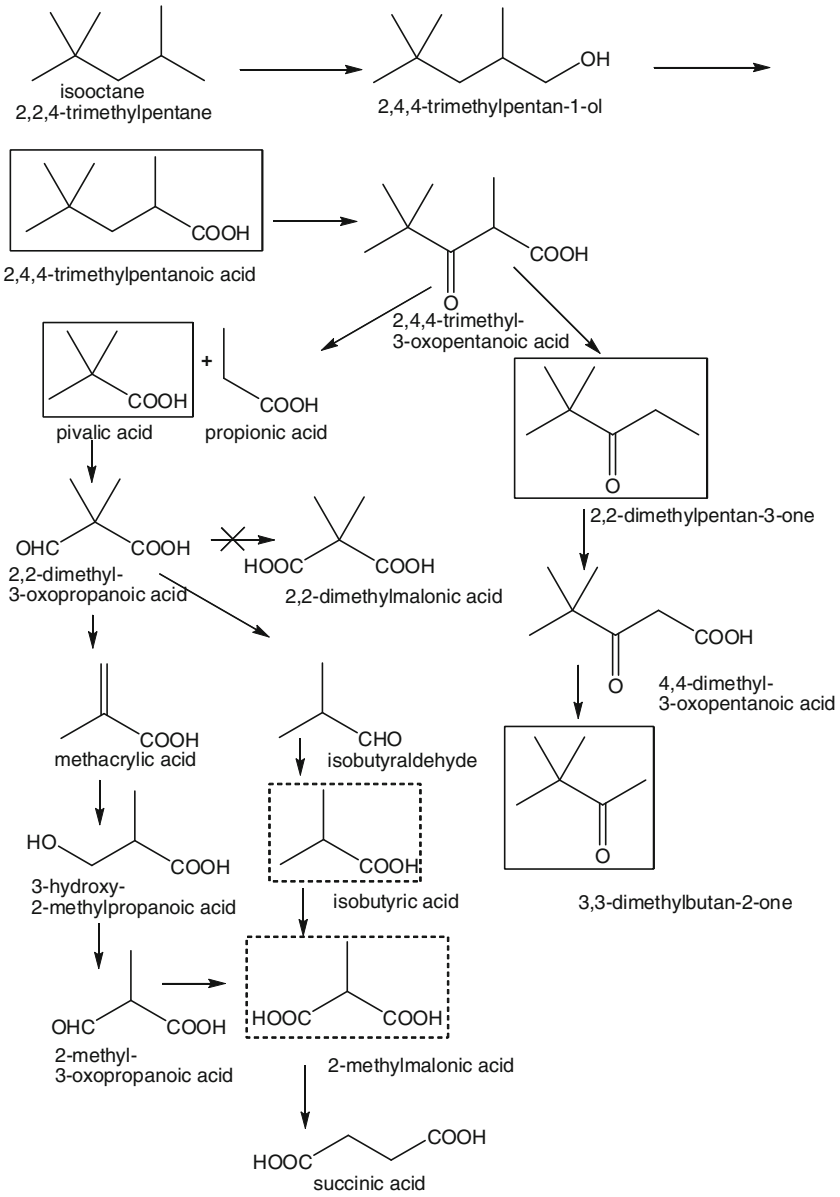

Fig. 4 Biodegradation of isooctane. The products isolated in cultures on isooctane are in *solid-line boxes* and proposed intermediates mineralized by isooctane-grown cells in *dotted-line boxes*. The free acid form is indicated for acids, but is meant to include CoA derivatives (Solano-Serena et al. 2004)

mechanisms have been proposed and partially documented for the degradation of compounds having a quaternary carbon atom in the molecule (see Fig. 3, which shows only the key steps of individual proposed mechanisms). The first involves hydroxylation of a methyl group to give diol; the second, a rearrangement of carboxyl by pivalyl-CoA mutase, and the third, a "rearrangement" of methyl group yielding a metabolite with tertiary carbon.

One of the possibilities of answering the question, whether pivalic acid is anthropogenic, can be the use of a combination of experiments published so far. As mentioned above, the enzymes *Gk*IcmF and *Cm*IcmF (Cracan and Banerjee 2012) can transform isovaleryl-CoA to pivalyl-CoA by 1,2-rearrangement. By using commercially available Leu isotopically labeled either by deuterium (L-leucine-5,5,5-D_3, L-leucine-D_{10}, and L-leucine-isopropyl-D_7) or by ^{13}C (L-leucine-3-^{13}C or L-leucine-$^{13}C_6$) or their combination (L-leucine-$^{13}C_6$,D_{10}) or isovaleric acid (α-ketoisovaleric acid-U-$^{13}C_5$,3-D_1 or α-ketoisovaleric acid-U-$^{13}C_5$), the enzyme reactions can be assumed to yield labeled pivalyl-CoA.

As shown in our previous study (Rezanka et al. 2011), deuterium-labeled pivalic acid can then be incorporated into fatty acids (genera *Alicyclobacillus*, *Rhodococcus*, and *Streptomyces*) and also into the antibiotic avermectin (*S. avermitilis*).

Bacteria suitable for the transformation can be found, for instance, among the species of the genus *Streptomyces* (e.g., *S. avermitilis* or *S. cinnamonensis*). They should include such species in which the fork of the polyketide pathway to primary and secondary metabolic pathways has been described. This would demonstrate the biosynthesis of compounds having in their molecule pivalic acid as a starter unit, show that pivalic acid is biosynthesized by some bacteria, and support the conclusion that pivalic acid is a natural compound.

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