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Pivalic acid acts as a starter unit in a fatty acid and antibiotic biosynthetic pathway in *Alicyclobacillus, Rhodococcus* and *Streptomyces*

Tomáš Řezanka,[1]* Lucie Siristova,[2] Olga Schreiberová,[2] Michal Řezanka,[3] Jan Masák,[2] Karel Melzoch[2] and Karel Sigler[1]

[1]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic.*

[2]*Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague, Czech Republic.*

[3]*Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague, Czech Republic.*

Summary

A biosynthetic pathway using pivalic acid as a starter unit was found in three bacterial species, *Alicyclobacillus acidoterrestris, Rhodococcus erythropolis* and *Streptomyces avermitilis*. When deuterium-labelled pivalic acid was added to *A. acidoterrestris* and *R. erythropolis* nutrient media it was incorporated into fatty acids to give rise to *tert*-butyl fatty acids (*t*-FAs). In addition, in *R. erythropolis*, pivalic acid was transformed into two starter units, i.e. isobutyric and 2-methylbutyric acid, which served as precursors of corresponding *iso*-even FAs and *anteiso*-FAs. In *S. avermitilis* the biosynthesis also yielded all three branched FAs; apart from this pathway, both pivalic and 2-methylbutyric acids were incorporated into the antibiotic avermectin.

Introduction

Natural products containing *tert*-butyl groups are in general not very frequent in nature (Dembitsky, 2006; Bisel *et al*., 2008). In some groups of natural products, however, these groups occur more frequently than in others. Natural substances containing *tert*-butyl group in the molecule are, e.g. peptides (hemiasterlin, milnamide A or discodermin E) having both enantiomers of the non-proteinogenic amino acid, i.e. *tert*-leucine. Other compounds containing *tert*-butyl are ginkgolides or many sterols with highly branched side-chains, usually obtained from marine sponges. Almost 300 of these compounds belonging to a few structural families are reported to have been isolated from natural sources (Dembitsky, 2006; Reaxys, 2010). However, from more than 200 compounds containing a *tert*-butyl group within Reaxys, 2010, which are described as 'Isolated Natural Products', a significant portion have been wrongly assigned in the data bank since they are in fact semisynthetic.

The biosynthesis of the *tert*-butyl group is still far from clear; for instance, a mevalonate pathway was described in the biosynthesis of ginkgolides (see the review by Bisel *et al*., 2008). According to another hypothesis, which has not yet been experimentally confirmed, an isobutyrate starter with subsequent methylation with *S*-adenosylmethionine by means of a free-radical mechanism can give rise to *tert*-valeryl or, in other words, pivaloyl polyketide starter unit (Bisel *et al*., 2008).

Considerable effort has been devoted to finding a suitable bacterium capable of biodegrading or biotransforming isooctane, i.e. *tert*-butyl group-containing 2,2,4-trimethylpentane, a common gasoline additive, or other 2,2-dimethylalkanes. Many bacteria, e.g. *Rhodococcus* (Auffret *et al*., 2009), *Mycobacterium* (Solano-Serena *et al*., 2000) or other bacteria such as *Acinetobacter, Arthrobacter, Bacillus* or *Gordonia* (Purswani *et al*., 2008), were found to degrade isooctane to CO_2 but the degradation pathway has not been elucidated. Only a few papers mention probable intermediates of the biodegradation. Thus, Solano-Serena and colleagues (2004) have reported on isooctane biodegradation via pivalic acid by *Mycobacterium austroafricanum*. Two pathways for the biotransformation of pivalic acid to other products have been proposed by Probian and colleagues (2003). The first was proposed to start with an anaerobic oxidation of a methyl group and degradation via dimethylmalonate to isobutyrate, which is the starting unit for the biosynthesis of *iso*-FAs. Activation of anaerobic oxidation of hydrocarbons often includes fumarate addition (Boll *et al*., 2002; Kniemeyer *et al*., 2007). Mbadinga and colleagues (2011) summarized other possible activation strategies. The second pathway should involve the activation

Received 14 December, 2010; accepted 13 February, 2011. *For correspondence. E-mail rezanka@biomed.cas.cz; Tel. (+420) 241 062 300; Fax (+420) 241 062 347.

to pivalyl-CoA and a rearrangement yielding 2-methylbutyryl-CoA, the starting unit of *anteiso*-FAs. This leads to our problem, viz. the biodegradation and/or biotransformation of pivalic acid.

This report is part of our investigation of precursor directed biosynthesis in bacteria within the framework of a comprehensive programme on the analysis and biosynthesis of lipids and natural compounds (Spizek and Rezanka, 2004; Lisa *et al*., 2007). We now report on the identification of a new metabolic pathway based on pivalic acid and on its incorporation into primary (fatty acids) and secondary metabolites (antibiotics avermectins).

Results and discussion

The bioremediation of isooctane (a common gasoline additive) to pivalic acid and further, via unknown intermediates, to carbon dioxide has been mentioned only once as a hypothesis (Solano-Serena *et al*., 2004). Probian and colleagues (2003) proposed the degradation of pivalic acid to isobutyrate and 2-methylbutyrate. To test these hypotheses, we added into a series of cultivations of different bacteria deuterium-labelled pivalic acid synthesized from commercially obtained deuterium-labelled *tert*-butyl chloride by Grignard reaction with CO_2; see *Experimental procedures*.

The model microorganisms used for these cultivations included three bacteria taxonomically sufficiently distant from each other. The members of genus *Alicyclobacillus* are Gram-positive, thermoacidophilic, strictly aerobic, heterotrophic, endospore-forming bacteria. The genus *Rhodococcus* belongs to aerobic, non-sporulating, non-motile Gram-positive bacteria with very large biosynthetic and bioremediation potential since it has a giant 9.7 megabase-pair genome (McLeod *et al*., 2006). *Streptomyces* are Gram-positive, and have also large genomes (more than 9 megabase pairs) (Ikeda *et al*., 2003). They are also the most frequently studied bacteria producing more than 1000 antibiotics (Watve *et al*., 2001). We cultivated the bacteria without and with deuterium-labelled pivalic acid. Since Probian and colleagues (2003) considered as intermediates in the degradation isobutyric and 2-methylbutyric acid, both of which are well-known precursors of branched chain fatty acids (Kaneda, 1991), we analysed the fatty acids in the first place.

Identification of fatty acids by GC-MS

Methyl esters have been the most analysed fatty acid derivatives and their chromatographic and mass spectrometric properties provide a wealth of information. However, the mass spectrometry of fatty acid methyl esters (FAMEs) with electron-impact ionization yields only limited information on the structure of fatty acids, especially branched-chain ones. Many natural branched-chain fatty acids were therefore identified as picolinyl esters by GC-MS, which reveals the fine differences between the spectra of the straight-chain FA.

The two most common branched-chain fatty acids are *iso*- or *anteiso*-FAs (*i*-FAs and *ai*-FAs respectively). Unfortunately, the methyl esters of both *i*- and *ai*-FA and also of other FA with different positions of methyl on the chain have very similar spectra (Apon and Nicolaides, 1975). For instance, methyl *i*-methylhexadecanoate exhibits in the mass spectrum always a perceptible $[M]^+$ ion and also $[M\text{-}15]^+$ ion at *m/z* 269 along with a doublet of ions for $[M\text{-}31]^+$ and $[M\text{-}32]^+$. Other important ions, i.e. $[M\text{-}65]^+$, $[M\text{-}55]^+$ and $[M\text{-}56]^+$, have abundances below 1% of base peak (ion at *m/z* 74) and can therefore be identified only with difficulty.

In *ai*-FA an ion at $[M\text{-}29]^+$ ($M\text{-}C_2H_5$) is more abundant than that equivalent to $[M\text{-}31]^+$ ($M\text{-}CH_3O$). Another ion that can be used is $[M\text{-}61]^+$, sometimes along with ions at $[M\text{-}60]^+$ and $[M\text{-}79]^+$.

Another problem concerning methyl esters is the fact that the mass spectrum of the model compound D_3-18:0 clearly shows that ions $[M\text{-}43]^+$ and $[M\text{-}29]^+$ do not involve loss of terminal methyl. The spectrum also contains the ion at *m/z* 43 but not that at *m/z* 46, which does not support simple cleavage of terminal three carbons. In addition, the presence of two ions at *m/z* 199 and at *m/z* 202 indicates that rearrangements and shifts of deuterium atoms along the chain take place – see the paper by Dinh-Nguyen (1968). All these findings have been confirmed in our study; see the spectrum of *t*-15:0, i.e. fatty acid having terminal *tert*-butyl group, i.e. neo-fatty acid.

The chromatography and mass spectrometry of *t*-FAs has been only sparsely studied. Two recent reviews marginally touching upon this problem (Dembitsky, 2006; Bisel *et al*., 2008) do not make reference to GC-MS. The only available study on the subject is from 1971 (Willecke and Pardee, 1971). The structure of methyl 12,12-dimethyltridecanoate (*t*-15:0) was derived based on molecular ion at *m/z* 256 and ions at *m/z* 200 $[M+H\text{-}t\text{-}Bu]^+$, and also ions $[M\text{-}15]^+$, $[M\text{-}31]^+$ and $[M\text{-}15\text{-}32]^+$. Mass spectrum of our deuterated FAME (D_9-*t*-15:0, Fig. 1) is much more complicated because it contains ions generated by fragmentation of the molecule containing atoms of deuterium and hydrogen.

Since all these features of methyl esters can stymie identification, we used picolinyl esters that exhibit typical prominent ions at *m/z* 92, 108, 151 and 164. The molecular ion is always abundant and it is always odd-numbered. Ions at *m/z* below 92 can be ignored.

Proceeding from the molecular ion, the first lower ion is formed by loss of methyl group $[M\text{-}15]^+$, followed by a series of ions 14 Da apart for loss of successive methylene groups. Rearrangement ions found in methyl esters

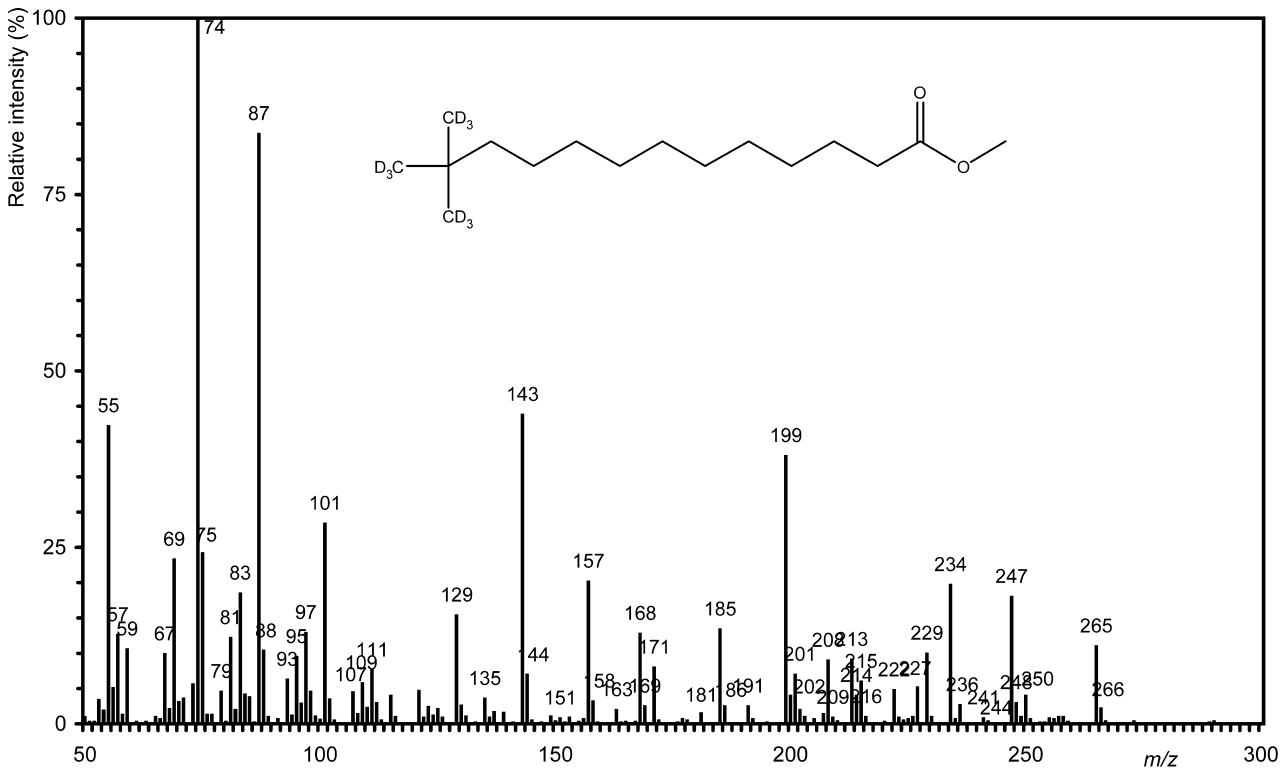


Fig. 1. Mass spectrum of labelled FAME D_9-*t*-15:0, The structure of methyl 12,12-dimethyltridecanoate with nine atoms of deuterium (D_9-*t*-15:0) was derived based on molecular ion at *m/z* 265 and ions at *m/z* 200 $[M+H\text{-}t\text{-}Bu\text{-}H]^+$, and also ions at *m/z* 247 $[M\text{-}18]^+$ and at *m/z* 234 $[M\text{-}31]^+$.

are not found here. In deuterium-labelled picolinyl octadecanoate acid, i.e. 17,18-dideutero stearic acid, $[M\text{-}16]^+$ ($M\text{-}CH_2D$) ion appears instead of $[M\text{-}15]^+$ and the further ion is $[M\text{-}31]^+$ ($M\text{-}C_2H_3D_2$); the following ions have already the classic 14 Da gap (http://lipidlibrary.aocs.org/ms/arch_pic/pi_sat/Pi0046.htm).

The mass spectrum of picolinyl *i*-FA (Fig. 2) resembles straight-chain saturated FA except for the very obvious gap of 28 Da between ions $[M\text{-}15]^+$ and $[M\text{-}43]^+$, which represents loss of ω-carbon and its attached methyl group. In the mass spectrum of picolinyl *ai*-FA (Fig. 3), this gap is shifted to between ions $[M\text{-}29]^+$ and $[M\text{-}57]^+$.

For picolinyl ester of *t*-FAs we assumed the presence of ion $[M\text{-}15]^+$ in a much higher intensity than in *i*-FA and naturally also in straight-chain FA. This was indeed found (Figs 4 and 5). The ratio of intensity of $[M]^+/[M\text{-}15]^+$ ions in straight-chain FA is about 3:1, in *i*-FA about 1:1 and in *t*-FA 1:3. The spectrum also contained an abundant (about 80% of base peak) ion $[M\text{-}57]^+$ (Figs 2 and 5). Such abundance of this ion is never reached in the *ai*-FA isomer.

Alicyclobacillus

Alicyclobacillus acidoterrestris was able to utilize and incorporate pivalic acid into its cellular lipids. The proportion of FAs labelled by deuterium reached 37% (Table 1). Labelled FAs were represented only by D_9-*t*-15:0 and D_9-*t*-17:0, neither labelled *i*- nor *ai*-FAs being found. The results show that the presence of pivalic acid in culture medium induced only a decrease of ω-alicyclic FAs (from 76.4% to 35.7%). The proportion of other FAs (*i*-, *ai*-, straight-chain, hydroxy and unsaturated) remained almost the same as in control cultivation.

The results show that *A. acidoterrestris* cannot transform pivalic acid into relevant primers (Fig. 1) in order to yield *i*- or *ai*-branched FAs. The presence of these unlabelled FAs is given by the bacterium utilizing different substrates from culture medium such as isobutyric, isovaleric and 2-methylbutyric acids (Kaneda, 1991). Branched-chain FA synthetase can utilize unchanged pivalic acid as a primer to synthesize unnatural *t*-FAs.

A side finding is that pivalic acid inhibited the growth of *A. acidoterrestris* in quite low concentrations. Initial tests with unlabelled pivalic acids showed a rapid decrease in bacterial growth with increasing pivalic acid concentration. The yield of dry biomass was 577, 127 and 19 mg l^{-1} of medium at 4.5, 5.0 and 10.0 mmol l^{-1} of unlabelled pivalic acid in culture medium respectively. One possible explanation may be related to the significant decrease of ω-alicyclic FAs (ω-FAs). The likely

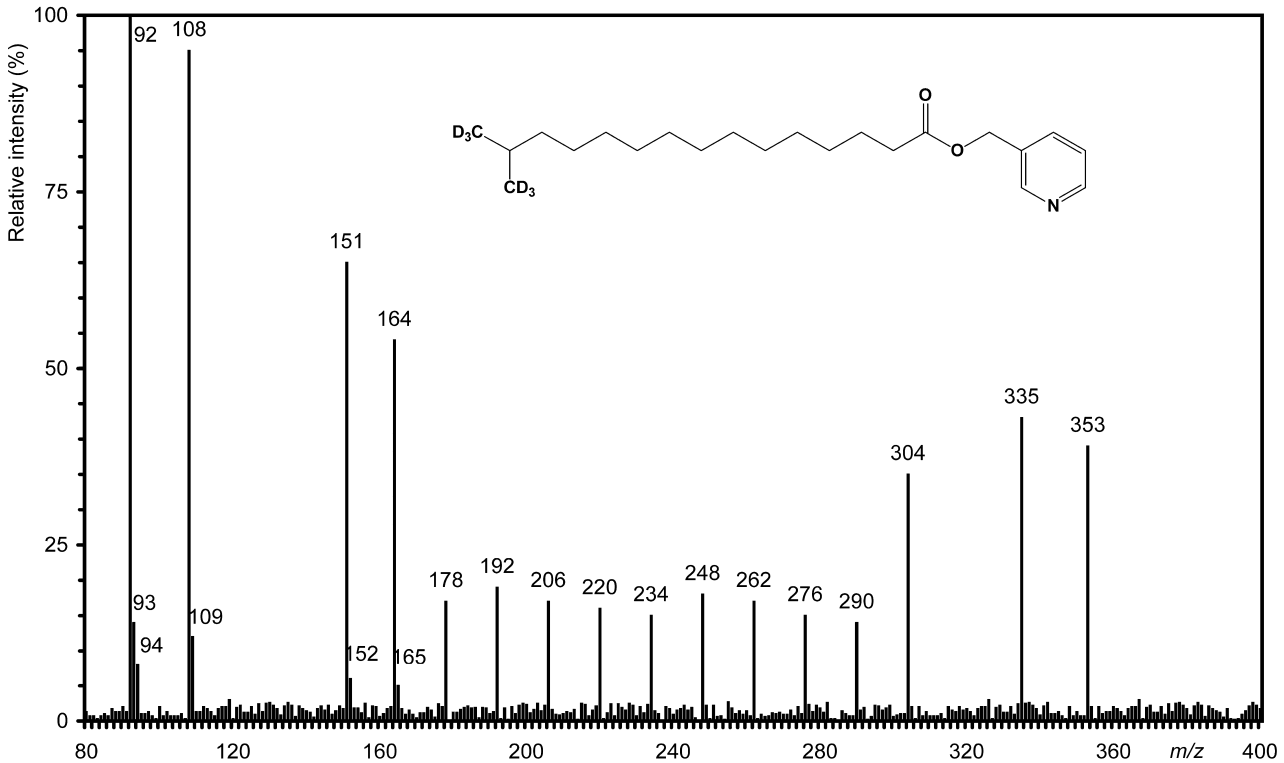


Fig. 2. Mass spectrum of labelled picolinyl ester D_6-*i*-16:0. The mass spectrum of picolinyl ester exhibits typical prominent ions at *m/z* 92, 108, 151 and 164. The molecular ion is at *m/z* 353, further ions are formed by loss of perdeutero methyl group, i.e. CD_3 $[M\text{-}18]^+$, followed by a series of ions 14 Da. An exception is the gap having 31 Da (CD_3CH) between ions at *m/z* 335 and *m/z* 304, affirmative of the structure of *iso*-picolinyl ester (D_6-*i*-16:0).

function of ω-FAs consists in providing resistance to higher temperature and acidic conditions (Kannenberg *et al*., 1984). The decrease in ω-FAs in our feeding experiment probably caused a decrease in resistance to acidic and thermal conditions and thus also in bacterial growth.

Rhodococcus

Lipids isolated from *Rhodococcus erythropolis* growing in a medium with pivalic acid contained branched *i-, ai-* and *t*-FAs (Table 1). The presence of deuterium in these substances has confirmed that the branched part comes from the deuterium-labelled pivalic acid or from its transformation products.

Degradation and/or transformation pathways of pivalic acid were proved in some microorganisms. Probian and colleagues (2003) isolated strains of denitrifying bacteria and suggested the presence of at least two transformation mechanisms of pivalate. The first one leads to oxidation of a methyl-group and subsequent decarboxylation of dimethylmalonate to isobutyrate. The second pathway involves activation by pivalyl-CoA ligase and a subsequent isomerization by pivalyl-CoA mutase providing 2-methyl-butyryl-CoA. Some reports indicate that the product of this pathway is 3-methyl-butyryl-CoA (isovaleryl-CoA) (Biocatalysis/Biodegradation Database, 2010).

Deuterium-containing *i*-FAs from *R. erythropolis* are even (D_6-*i*-16:0; D_6-*i*-18:0) but *ai*- and *t*-FAs are odd (D_8-*ai*-13:0; D_8-*ai*-15:0; D_8-*ai*-17:0; D_9-*t*-15:0; D_9-*t*-17:0). None of these fatty acids was synthesized in the medium without pivalic acid. Based on the results, it could be assumed that pivalic acid enters into even *i*-FAs after conversion to dimethylmalonate and subsequent decarboxylation to isobutyrate.

Because odd *i*-FAs were not identified, it is evident that the *R. erythropolis* pivalyl-CoA mutase does not provide 3-methyl-butyryl-CoA.

Odd *t*-FAs are created by a direct entry of activated pivalic acid (pivalyl-CoA). 2-Methylbutyrate, the product of pivalic acid isomerization (see above), is the intermediate of odd *ai*-FAs. *Rhodococcus erythropolis* preferred reactions for pivalic acid entry into the fatty acid metabolism. The total amount of *ai*- and *t*-FAs from pivalic acid represents 26.7% of the determined fatty acids. However, fatty acids containing isobutyrate, originating from pivalic acid, occur only at 5.6%.

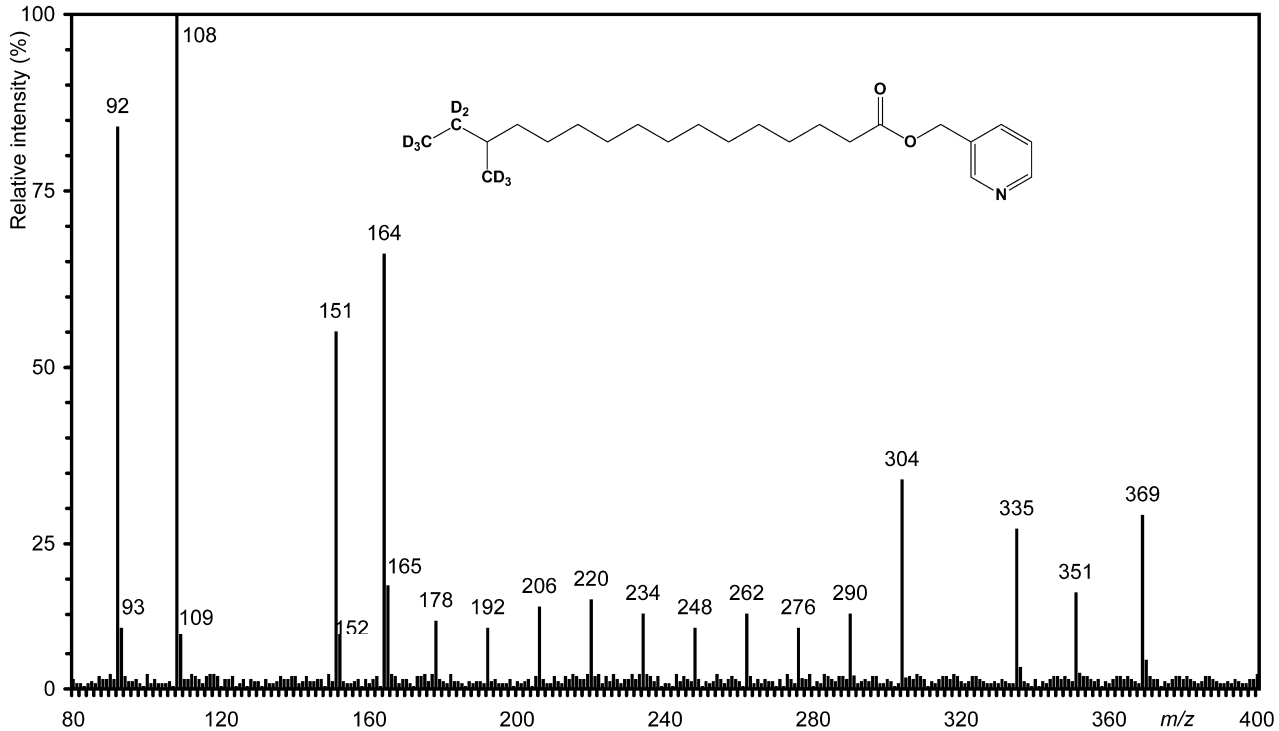


Fig. 3. Mass spectrum of labelled picolinyl ester D_8-*ai*-17:0. The molecular ion is at *m/z* 369, further ions are formed by loss of perdeutero methyl group, i.e. CD_3 $[M-18]^+$ and perdeutero ethyl group CD_3CD_2 $[M-44]^+$ followed by a series of ions 14 Da. An exception is the gap having 31 Da (CD_3CH) between ions at *m/z* 335 and *m/z* 304, affirmative of the structure of *anteiso*-picolinyl ester (D_8-*ai*-17:0).

Streptomyces – *fatty acids*

The spectrum of fatty acids in *Streptomyces avermitilis* was the most complex of all three bacteria under study, including all three types of labelled FAs, i.e. *t*-, even-*i*- and *ai*-FAs. The analysis of *S. avermitilis* was complicated by the fact that this species synthesizes both even- and odd-numbered *i*-, *ai*- and straight-chain FA including unsaturated ones (Hopwood and Sherman, 1990).

As compared with the control cultivation, a qualitative increase in the number of identified FA (from 16 to 22) and marked changes in their quantitative proportions took place (Fig. 6). Table 1 shows that deuterium-labelled pivalic acid was incorporated into FAs to a level of about 10% of total FA. On the other hand, the content of straight chain FA sharply dropped to about one-third, odd-*i*-FA to one-fourth and unsaturated FA dropped to less than one half. Further, several specific trends appeared: even *iso* unlabelled FA showed a weak to negligible decrease from 17.6% to 15.9% total FA whereas labelled *i*-even FAs reached 80% of unlabelled FA. A similar situation was observed in *ai*-FA; the content of total *ai*-FA increased while that of unlabelled *ai*-FA decreased, its level dropping below that of labelled FA (25.1 and 20.0% total FA respectively). These values attest to the efficiency of this biosynthetic pathway (pathway 3, see Fig. 7), which was further confirmed also in the case of secondary metabolites, i.e. avermectins produced by *S. avermitilis*.

Streptomyces – *avermectins*

The naturally occurring avermectins, metabolic products of *S. avermitilis*, are a series of 16-membered macrocyclic lactone derivatives with potent anthelmintic and insecticidal properties. Eight different avermectins were isolated with a major (a-component) and a minor (b-component) component, usually in ratios of 80:20 to 90:10 (Ikeda and Omura, 1997); see Fig. 8 for the structure of A_2a.

Biosynthesis of avermectins has been studied many times (Ikeda and Omura, 1995; 1997). The 2-methylbutyryl side-chain of the 'a' components and the isobutyryl side-chain of the 'b' components are biosynthesized from L-isoleucine or L-valine via branched-chain 2-oxo acids. Novel avermectin derivatives were prepared by precursor directed biosynthesis by Dutton and colleagues (1991). The authors used tens of 'unnatural' starter units and identified appropriate avermectin derivatives. Based on these data we also used an unnatural starter unit, i.e. deuterium-labelled pivalic acid, and identified the corresponding avermectin, having on carbon 25 not only isopropyl or 2-methylbutyryl but also *t*-butyl side-chain.

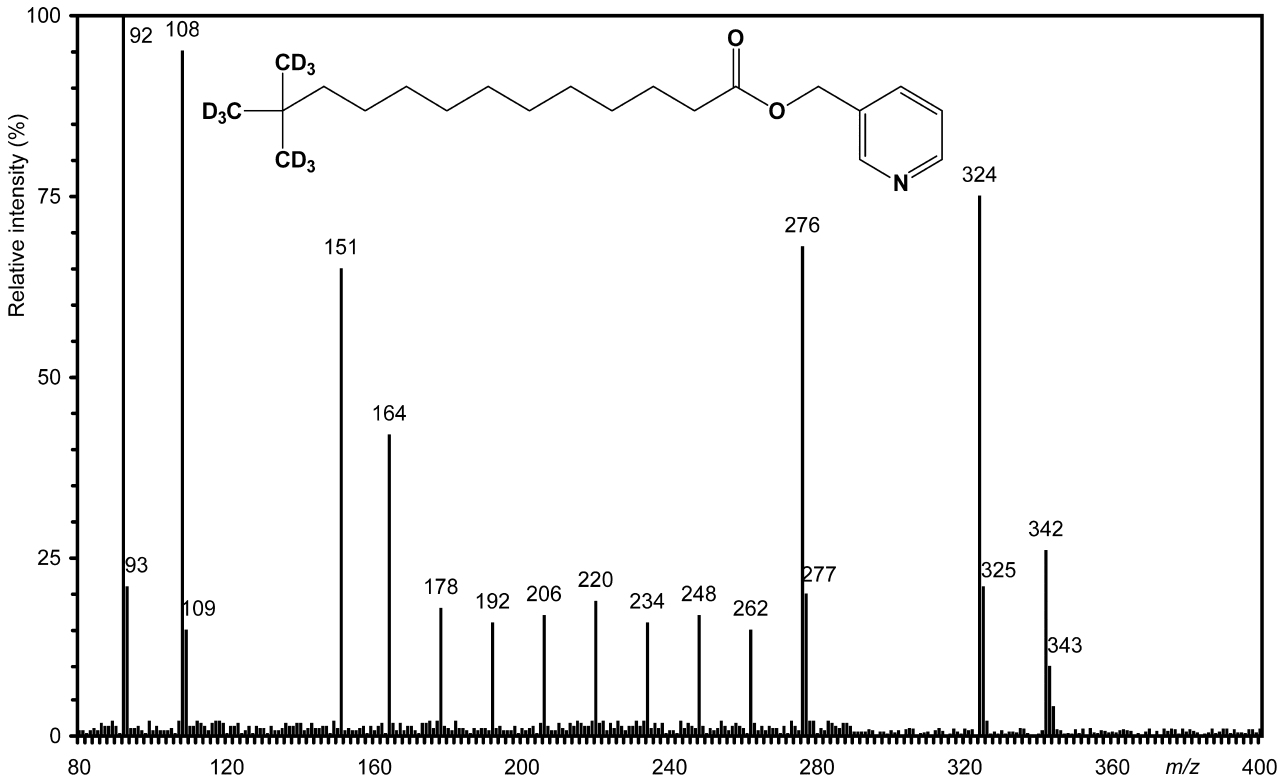


Fig. 4. Mass spectrum of labelled picolinyl ester D_9-*t*-15:0. The molecular ion is at *m/z* 342, further ion is formed by loss of perdeutero methyl group, i.e. CD_3 $[M\text{-}18]^+$. The intensity of $[M\text{-}CD_3]^+$ ion increases with the number of terminal methyls. The following peaks have a series of ions 14 Da. An exception is the gap having 31 Da (CD_3CH) between ions at *m/z* 335 and *m/z* 304, affirmative of the structure of terminal *tert*-butyl, i.e. picolinyl ester (D_9-*t*-15:0).

The strain of *S. avermitilis* was cultivated under previously described conditions (Cimburkova *et al*., 1988), see also *Experimental procedures*. Sodium salt of deuterium-labelled pivalic acid (its synthesis is described below) was added at the beginning of cultivation, the biomass was centrifuged and extracted with a mixture of solvents.

The extracted mixture of avermectins was separated by semipreparative RP-HPLC. However, neither the semipreparative nor an analytical RP-HPLC, in which the efficiency of two connected columns was about 30 000 plates per 30 cm, succeeded in separating labelled and unlabelled avermectins. To determine the incorporation of deuterium-labelled compounds we therefore used two mutually independent methods, i.e. ^{2}H-NMR and mass spectrometry.

^{2}H-NMR is a unique tool for detecting the sites of modification of a molecule caused by introducing ^{2}H-labelled compounds because the observed peaks are necessarily singlets due to negligible ^{1}H/^{2}H and ^{2}H/^{2}H coupling. The chemical shifts of ^{2}H-NMR can be compared directly to those of the corresponding ^{1}H-NMR, making the assignments straightforward. The spectrum of the compounds from A_2a peak showed that this peak is a mixture of two deuterium-labelled compounds. The chemical shifts δ_D at 0.94, 1.01 and 1.47 ppm prove the presence of methyl and methylene units, the intensity of the signals of methyl groups (δ_D 0.94 and 1.01) being at least 1:4; this points to the presence of both *sec*-butyl and *t*-butyl side-chains.

The high-resolution mass spectrum of compounds from the peak A_2a contained pseudomolecular ions $[M+Na]^+$, i.e. ions in the interval of 927–930 Da and in the interval of 935–939 Da (Fig. 9). The ion at *m/z* 927.5080 had the formula $C_{49}H_{76}NaO_{15}$, which corresponds to avermectin A_2a.

Ion at *m/z* 935.5586 had the formula $C_{49}H_{68}D_8NaO_{15}$; this enabled us to determine the rearrangement of labelled pivalic acid to labelled 2-methylbutyric acid (pathway 3, Fig. 7) and its incorporation as a starter unit into avermectins. This proved again the biosynthesis of avermectin A_2a, in this case with side-chain labelled by eight atoms of deuterium.

The role of labelled pivalic acid as a starter unit in the biosynthesis of avermectin was confirmed again by HR-MS; see the inset in Fig. 9. The HR-MS of ion at *m/z* 936 provided two ions, first at *m/z* 936.5620 with the summary formula $C_{48}{}^{13}CH_{68}D_8NaO_{15}$ (this was the isotopic ion belonging to avermectin A_2a) and the second ion at *m/z* 936.5647 with the summary formula $C_{49}H_{67}D_9NaO_{15}$.

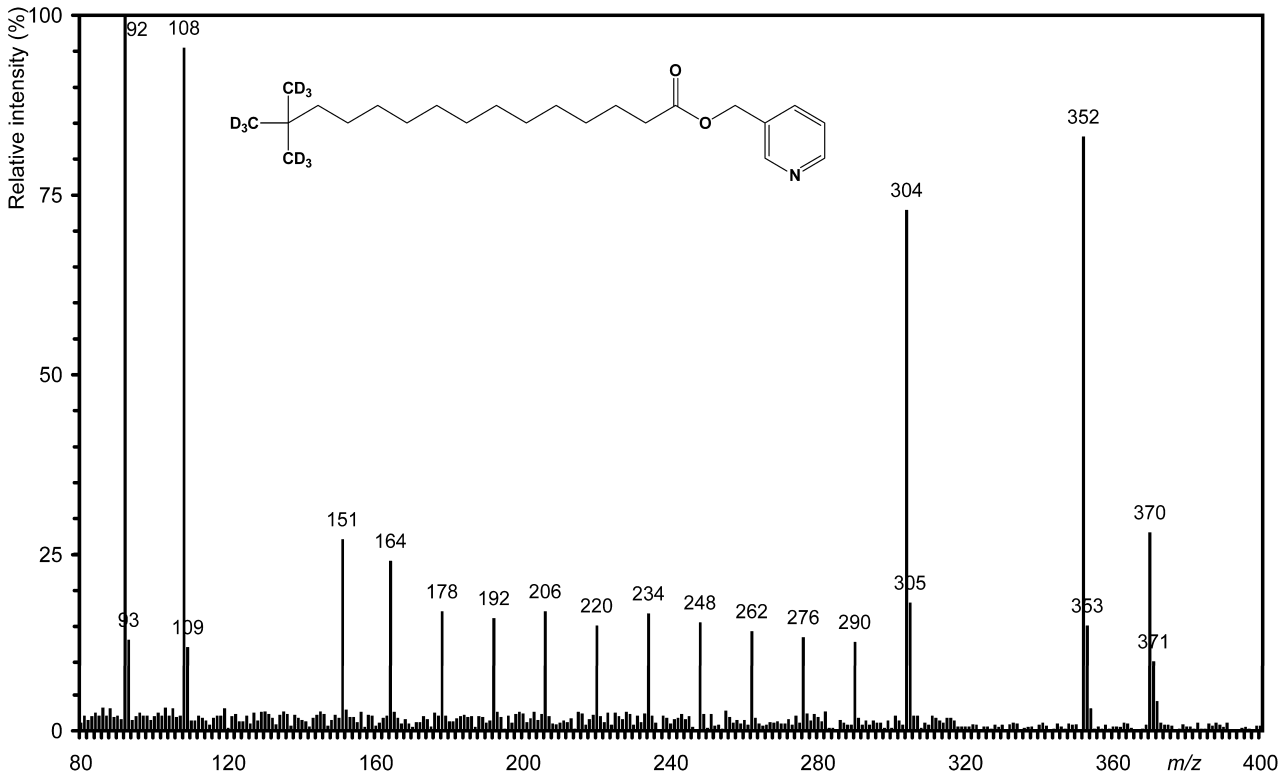


Fig. 5. Mass spectrum of labelled picolinyl ester D_9-*t*-17:0. This compound has the molecular ion at *m/z* 370, further values of important ions are shifted by 28 Da to higher values relative to D_9-*t*-15:0.

The presence of this ion confirmed that deuterium-labelled pivalic acid is a starter unit in the biosynthesis of avermectins, giving rise to an as yet undescribed avermectin, i.e. isomer of A_2a. This has extended the possible number of precursors described by Dutton and colleagues (1991), and has proved that biosynthetic pathway 3 (Fig. 7) holds not only for the biosynthesis of a fatty acid (see above) but also for the biosynthesis of secondary metabolites, i.e. avermectins.

The bioconversion of pivalic acid to isobutyric acid (biosynthetic pathway 2, Fig. 7) and its incorporation into avermectins has not yet been observed since the mutant made at our institute produces avermectins of group b (precursor Val) only in minimum amounts.

To confirm the incorporation of labelled pivalate into avermectins, we also measured HR-EI-MS of the compounds from chromatographic peak A_2a; the mass spectrum here exhibits the presence of many ions (see Albers-Schönberg *et al*., 1981). Ion at *m/z* 169 (for structure of this ion, called ion m in the paper of Albers-Schönberg *et al*., 1981, see also Table 2) is an ion with the lowest mass in which the structure of the side-chain can still be determined. High-resolution mass spectrum shows the presence of six ions having the structure of the m ion; see Table 2. This again confirmed both the direct incorporation of labelled pivalic acid and its rearrangement to 2-methylbutyric acid, and the incorporation of both these acids as starter units in the biosynthesis of the avermectins.

Conclusion

We showed that in all the three bacteria deuterium-labelled pivalic acid is incorporated and serves as a starter unit for the biosynthesis of *t*-FAs. Biosynthesis of *t*-FAs from sodium pivalate has so far been described only in *Bacillus subtilis* (Willecke and Pardee, 1971) and our results show that the incorporation of pivalate and biosynthesis of corresponding fatty acids is more widespread in bacteria.

Two of the bacteria, *R. erythropolis* and *S. avermitilis*, convert pivalic acid to isobutyric and 2-methylbutyric acid, which are then used as starter units for the biosynthesis of primary metabolites such as *i*-even FAs and *ai*-FAs. In *S. avermitilis* both pivalic and 2-methylbutyric acid were incorporated into the antibiotic avermectin to yield avermectin t that has not yet been described. The conversion of pivalic acid to isobutyric and 2-methylbutyric acid opens a potential way to the bioremediation of exogenous pivalic acid coming from prodrugs (Probian *et al*., 2003) or from

Table 1. Fatty acids from *A. acidoterrestris, R. erythropolis* and *S. avermitilis*.

			Rhodococcus		*Alicyclobacillus*		*Streptomyces*	
Peak No.[a]	Fatty acid	ECN[b]	Control	With pivalate	Control	With pivalate	Control	With pivalate
1	D_8-*ai*-13:0	12.67	–[c]	2.9	–	–	–	–
2	*ai*-13:0	12.70	–	–	–	–	0.8	0.9
3	D_6-*i*-14:0	13.60	–	–	–	–	–	3.3
4	*i*-14:0	13.63	–	3.7	–	–	3.2	2.9
5	14:0	14.00	9.8	5.4	–	0.1	1.5	0.4
6	*t*-15:0	14.32	–	4.3	–	23.8	–	3.9
7	*i*-15:0	14.63	–	–	0.8	0.7	5.1	1.6
8	D_8-*ai*-15:0	14.68	–	8.6	–	–	–	18.4
9	*ai*-15:0	14.71	–	–	0.9	0.8	27.4	14.0
10	15:0	15.00	8.2	4.7	0.7	0.7	4.2	1.6
11	D_6-*i*-16:0	15.61	–	4.2	–	–	–	9.5
12	*i*-16:0	15.64	–	–	1.1	1.2	14.4	13.0
13	16:1	15.80	18.5	17.5	–	0.1	5.7	2.4
14	16:0	16.00	19.3	9.1	8.7	7.8	22.9	7.6
15	*t*-17:0	16.34	–	5.2	–	18.2	–	7.2
16	*i*-17:0	16.65	–	–	1.3	1.4	1.8	0.2
17	D_8-*ai*-17:0	16.70	–	5.7	–	–	–	6.7
18	*ai*-17:0	16.73	–	–	3.4	3.4	9.6	5.1
19	17:1	16.81	9.2	6.8	–	–	1.3	0.8
20	17:0	17.00	4.9	3.5	1.1	1.0	1.1	0.3
21	ω-cyc-17:0	17.61	–	–	60.6	27.5	–	–
22	D_6-*i*-18:0	17.64	–	1.4	–	–	–	–
23	*i*-18:0	17.67	–	–	–	–	–	–
24	18:1	17.73	27.0	14.1	–	0.2	0.8	0.2
25	18:0	18.00	3.1	2.9	1.0	0.8	0.2	–
26	3-OH-17:0	18.51	–	–	4.6	4.1	–	–
27	ω-cyc-19:0	19.63	–	–	15.8	8.2	–	–
	Even-*i*		0.0	9.3	1.1	1.2	17.6	28.7
	Odd-*i*		0.0	0.0	2.1	2.1	6.9	1.8
	ai		0.0	17.2	4.3	4.2	37.8	45.1
	n		45.3	25.6	11.5	15.4	29.9	9.9
	Monoenoic		54.7	38.4	0.0	0.3	7.8	3.4
	Cyclic		0.0	0.0	76.4	35.7	0.0	0.0
	OH		0.0	0.0	4.6	4.1	0.0	0.0
	t		0.0	9.5	0.0	37.0	0.0	11.1
	Even-*i*		0.0	9.3	1.1	1.2	17.6	28.7

a. See Fig. 6.
b. Equivalent chain number.
c. Minority FAs up to 0.1% of total FAs were omitted.

the gasoline additive isooctane. The biosynthetic pathway was discovered by employing state-of-the-art analytical techniques such as ^{2}H-NMR, HR-FAB-MS and GC-MS, and by using intentionally synthesized starter unit, i.e. deuterium-labelled pivalic acid.

Experimental procedures

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 76.86 MHz (^{2}H). The data were then acquired on the avermectin A_2a sample dissolved in ^{2}H-depleted H_2O with broadband proton decoupling. Typically, the number of scans required per run for each sample was about 10 000. All the spectra were acquired at 25°C with sweep widths of 1.5 kHz for ^{2}H. High- and also low-resolution mass spectra were recorded using a VG 7070E-HF spectrometer. HR-FAB-MS (positive and/or negative ion mode) were obtained with a PEG-400 matrix.

The synthesis of deuterium-labelled sodium pivalate was adapted from a previously described synthesis of pivalic acid (Puntambeker *et al.*, 1941). Briefly, into a dry 100 ml three-necked flask were added magnesium turnings (1.2 g, 50 mmol) and diethyl ether (4 ml). Then a solution of D_9-*t*-butylchloride (5.0 g, 41.91 mmol) in 19 ml of diethyl ether was added dropwise under argon atmosphere. The mixture was refluxed for 1 h, and then cooled to 0°C and 100-fold excess of carbon dioxide was bubbled through the mixture. Carbon dioxide was generated from dry ice and dried by bubbling through sulfuric acid. The mixture was then hydrolysed with 5% hydrochloric acid, and then extracted twice with ether. Combined organic layers were dried over Na_2SO_4, and evaporated under vacuum giving D_9-pivalic acid as a colourless liquid; this was then transformed into sodium salt by addition of one equivalent sodium hydroxide in water and by subsequent evaporation under reduced pressure. Reaction was carried out three times. All three reactions yielded 5.95 g (36%) of the $(CD_3)_3COONa$ as a white powder. MS (ESI): $m/z = 110.2$ $[M\text{-}Na]^-$.

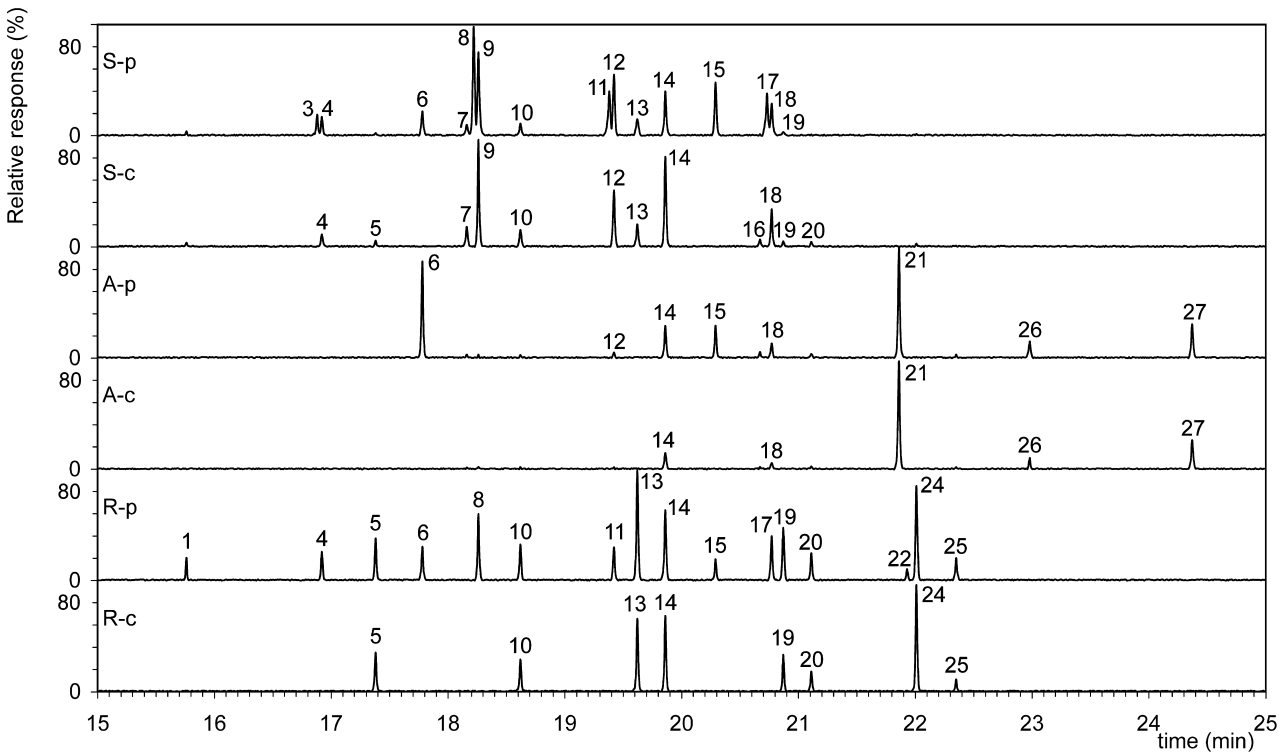


Fig. 6. GC-MS of FAMEs from *A. acidoterrestris, R. erythropolis* and *S. avermitilis*. R-c (*R. erythropolis* – control cultivation), R-p (cultivation with labelled sodium pivalate), A-c (*A. acidoterrestris* – control cultivation), A-p (cultivation with labelled sodium pivalate), S-c (*S. avermitilis* – control cultivation), S-p (cultivation with labelled sodium pivalate). Individual numbers at peaks are identified FAME. For their formulas (short-hand notation) see Table 1.

Strain and cultivation

Alicyclobacillus acidoterrestris CCM 4660 was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic) and it was cultivated in an alicyclobacillus medium (Siristova *et al*., 2009) containing (g l^{-1}): yeast extract 6.0, glucose 5.0, $CaCl_2 \cdot 2H_2O$ 0.25, $MgSO_4 \cdot 7H_2O$ 0.5, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 3.0 and 1 ml l^{-1} of trace element solution in g l^{-1}: $ZnSO_4 \cdot 7H_2O$ 0.1, $MnCl_2 \cdot 4H_2O$ 0.03, H_3BO_3 0.3, $CuCl_2 \cdot 6H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.01, $NiCl_2 \cdot 6H_2O$ 0.02, $Na_2MoO_4 \cdot 2H_2O$ 0.03; pH was adjusted to 4. Batch cultivation was carried out under aerobic conditions in 500 ml Erlenmeyer flasks filled with 200 ml of medium with the addition of 500 mg l^{-1} of labelled sodium pivalate. Erlenmeyer flasks were placed in a temperature-controlled shaking device (Siristova *et al*., 2009). Cultivation proceeded for 24 h, at 45°C and 200 r.p.m. After cultivation, the biomass was concentrated by centrifugation (10 000 r.p.m.) and lyophilized.

Cells of *R. erythropolis* CCM 2595 were grown in Erlenmeyer flasks using a rotary shaker (100 r.p.m., 30°C) in basic mineral medium (g l^{-1}): KH_2PO_4 0.17; $MnCl_2 \cdot H_2O$ 0.001; K_2HPO_4 0.13; $CaCl_2 \cdot 2H_2O$ 0.00026; $(NH_4)_2SO_4$ 0.71; $FeSO_4 \cdot 7H_2O$ 0.0006; $MgCl_2 \cdot 6H_2O$ 0.34; $Na_2MoO_4 \cdot 2H_2O$ 0.002, containing deuterium-labelled sodium pivalate (3 g l^{-1}) as carbon source and 1 g l^{-1} sodium succinate as a co-substrate; pH was adjusted to 7.0. The initial biomass concentration was adjusted to optical density 0.3 measured at 400 nm. The cells were collected in late exponential phase (72 h) by centrifugation (9050 g, 10 min), washed twice with physiological solution and lyophilized (dry biomass yield 238 mg l^{-1}).

The mutant strain *S. avermitilis* C-18 was obtained by genetic improvement (Cimburkova *et al*., 1988) of ATCC 1267. The inoculum was cultivated for 1 day in a complex medium containing (g l^{-1}): glucose 5; soybean meal 15; and yeast extract 5; fermentation flasks were inoculated with 2% of the inoculum (1 day old) and cultivated for 10 days. The following synthetic production medium (g l^{-1}) was used for cultivation: glucose 30, $CaCO_3$ 5, NaCl 2, K_2HPO_4 0.5, $FeSO_4 \cdot 7H_2O$ 0.05, $MnSO_4 \cdot 7H_2O$ 0.05, $ZnSO_4 \cdot 7H_2O$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.1. The cultivation was performed in 500 ml round-bottom flasks with or without the addition of 300 mg l^{-1} of labelled sodium pivalate on a rotary shaker (2.8 r.p.m.) at 28°C.

Identification of avermectins

Avermectins in the broth were determined by HPLC (Omura *et al*., 1991). The mycelia were harvested by centrifugation at 3000 r.p.m. for 5 min, the mycelial pellet was extracted with acetone (10 ml) followed by dichloromethane (10 ml) and the organic phase was separated, filtered and evaporated to dryness. The residue was taken up in methanol (0.5 ml) ready for HPLC preparation. The liquid chromatograph was the semipreparative Gradient LC System G-1

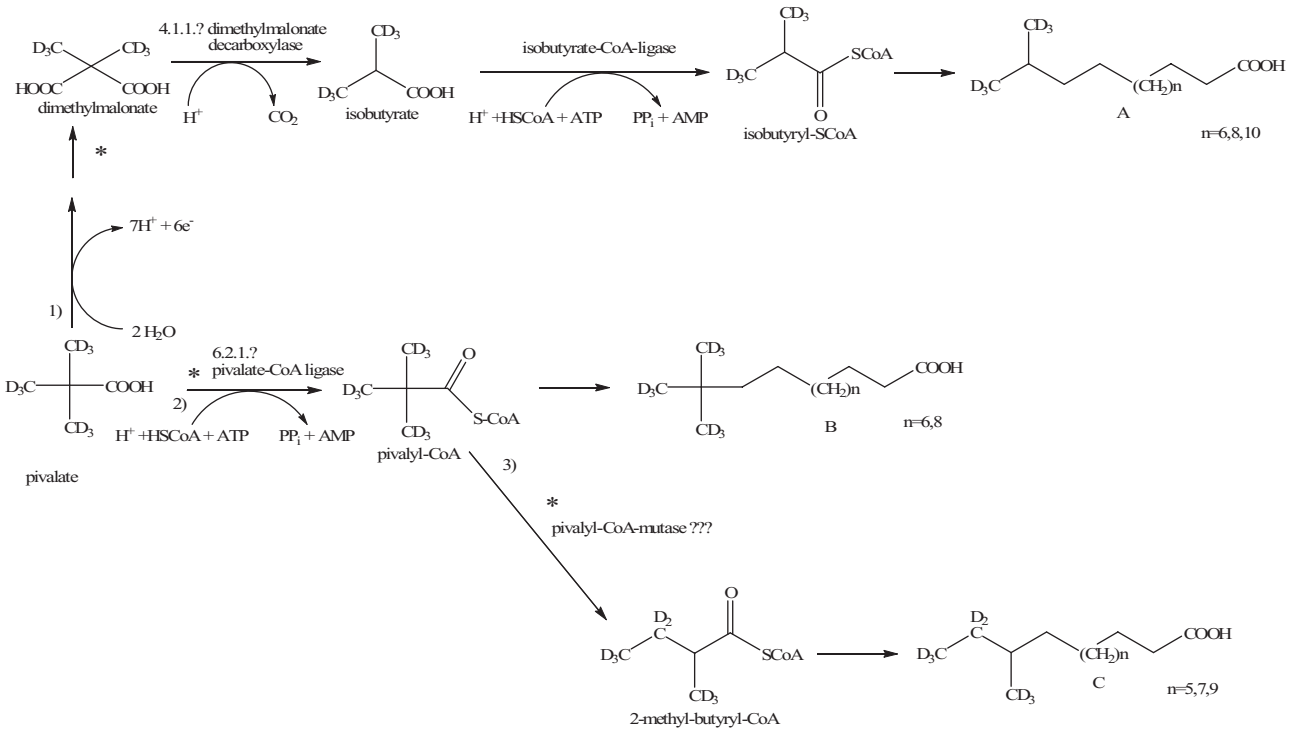


Fig. 7. Proposed metabolic pathway of deuterium-labelled sodium pivalate. Individual biosynthetic pathways are marked with the potential enzymes, possible coenzymes and low-molecular products. The pathways proposed by Probian and colleagues (2003) are marked with asterisks (*). ? EC number is not unambiguously determined. ??? Catalysis by this enzyme is only presumed.

(Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml min^{-1}), an SCL-6A system controller, an SPD ultraviolet detector an SIL-1A sample injector and a C-R3A data processor, with preparative Supelcosil® LC-18 HPLC column 5 μm particle size, L × I.D. 25 cm × 21.2 mm with mobile phase containing acetonitrile–methanol–water (62:18:20 v/v/v) to acetonitrile–methanol–water (31:9:60 v/v/v) for 40 min, a flow rate of 9.9 ml min^{-1}, and monitored by a variable wavelength detector at 246 nm (HP 1040M Diode Array Detector).

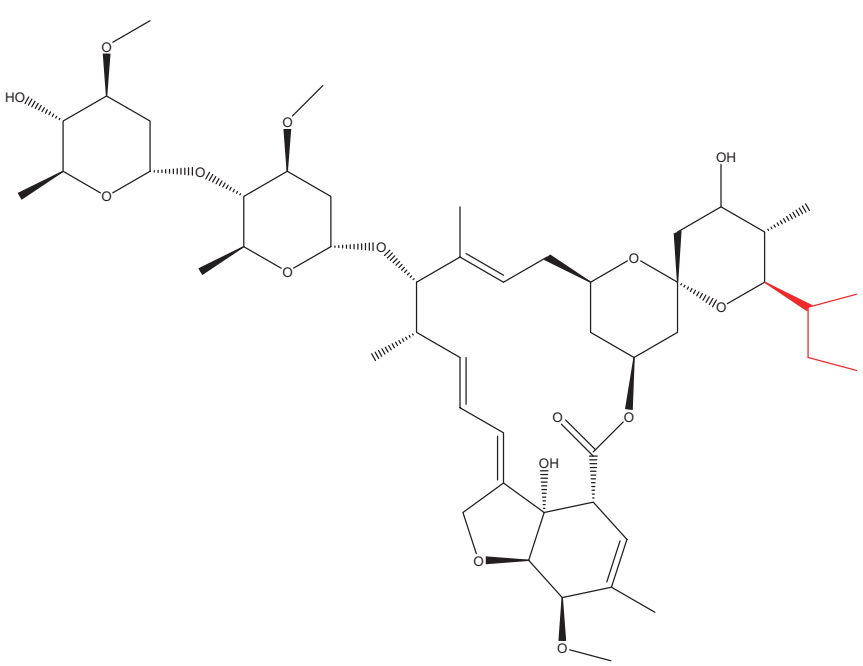


Fig. 8. The structure of avermectin A_2a. Side-chain is the site at which the starter unit is incorporated into the avermectin molecule.

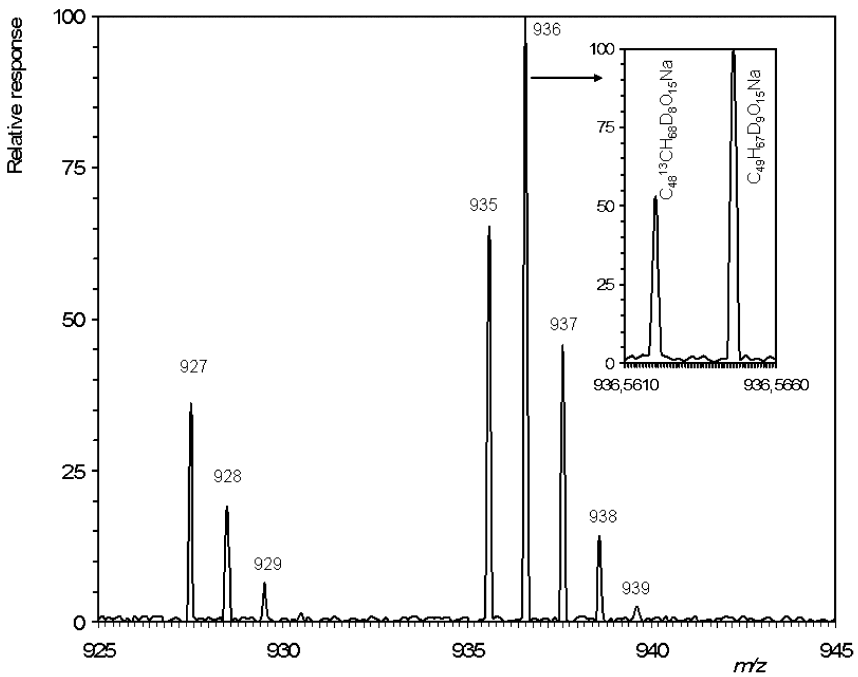
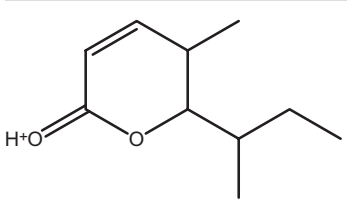
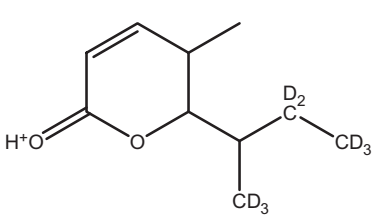
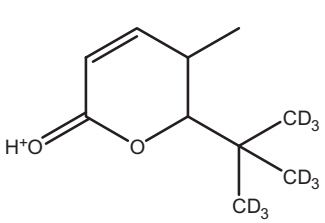


Fig. 9. HR-FAB-MS of a mixture of avermectins after HPLC. The mass spectrum contains pseudomolecular ions $[M+Na]^+$, i.e. ions in the interval of 927–930 Da and in the interval of 935–939 Da. The inset shows HR-MS of two ions at *m/z* 936.5620 with the summary formula $C_{48}{}^{13}CH_{68}D_8NaO_{15}$ (this was the isotopic ion belonging to avermectin A_2a) and the second ion at *m/z* 936.5647 with the summary formula $C_{49}H_{67}D_9NaO_{15}$. The presence of this ion confirmed that deuterium-labelled pivalic acid is a starter unit in the biosynthesis of avermectins.

Table 2. Structure and some characteristics of **m** ion.

Structure	*m/z*	Formula	Δ ppm	Intensity (in %)
	169.1228	$C_{10}H_{17}O_2$		82
-″-	170.1262	$C_{10}{}^{13}CH_{17}O_2$		8
	177.1732	$C_{10}H_9D_8O_2$	+0.1	100
-″-	178.1766	$C_{10}{}^{13}CH_9D_8O_2$	+0.2	10
	178.1794	$C_{10}H_8D_9O_2$	+0.1	69
-″-	179.1828	$C_{10}{}^{13}CH_8D_9O_2$	+0.1	7

Two Ascentis® Express C18 HPLC Columns (2.7 μm particle size, L × I.D. 15 cm × 3.0 mm; Supelco, Prague) in series were used for analytical determination of purity separated peaks. This set-up provided us with a high-efficiency column – approximately 30 000 plates per 30 cm. LC was performed at a flow rate of 300 μl min^{-1} with a linear gradient of mobile phase (see above) for 40 min. The flow (0.37 ml min^{-1}) was monitored by a variable wavelength detector at 246 nm.

Avermectin A_2a HRFABMS (*m/z*): 935.5586 $[M+Na]^+$, calculated for $[C_{49}H_{68}D_8O_{15}Na]^+$ 935.5584; $[M+Na+1]^+$ 936.5620 calculated for $[C_{48}{}^{13}CH_{68}D_8NaO_{15}]^+$ 936.5618; Avermectin t 936.5647 $[M+Na]^+$, calculated for $[C_{49}H_{67}D_9NaO_{15}]^+$ 936.5646.

Analysis of fatty acids

The extraction procedure was based on the method of Bligh and Dyer (1959). The alcohol–water mixture was cooled, one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

The lipids were reacted with 3% HCl in methanol at 80°C for 1 h. FAMEs were extracted with 2 ml of hexane, dried and analysed.

Gas chromatography-mass spectrometry of FAME was performed on a GC-MS system consisting of Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization and CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 μm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature programme: 5 min at 50°C, increasing at 10°C min^{-1} to 320°C and 15 min at 320°C. Helium was the carrier gas at a flow of 0.52 ml min^{-1}. All spectra were scanned within the range *m/z* 50–600.

Picolinyl esters of FA were prepared according to Rezanka (1990). A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 ml, 1.0 M) was added to nicotinyl alcohol (1 ml). After mixing, the appropriate fatty acid methyl esters (~0.5 mg) in dry dichloromethane (1 ml) were added, and the mixture was held at 40°C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate and evaporated.

A fatty acid picolinyl ester mixture was analysed on the instrument described above. Injection temperature (splitless injection) was 100°C, and an Ultra-1 capillary column was used. The temperature programme was as follows: 100°C for 1 min, subsequently increasing at 20°C min^{-1} to 180°C and at 2°C min^{-1} to 280°C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm s^{-1}. All spectra were scanned within the range *m/z* 70–650.

Acknowledgements

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