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ORIGINAL PAPER

Factors influencing cell fatty acid composition and A40926 antibiotic complex production in *Nonomuraea* sp. ATCC 39727

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Abstract A40926 is a glycopeptide antibiotic complex consisting of several structurally related factors. It is produced by fermentation of *Nonomuraea* sp. ATCC 39727 and the complex components differ in the structure of the fatty acid moiety linked to the aminoglucuronic acid unit. In previous work, we observed that the production of single factors in glycopeptide antibiotic complexes could be selectively enhanced by the addition of suitable precursors to the culture medium. In this contribution, we examine the effects of branched amino acid addition to fermentation of *Nonomuraea* sp. in a chemically defined minimal medium. The changes in the composition of cell fatty acids correlate to the fatty acid distribution within the A40926 complex in diverse cultivation conditions. *Nonomuraea* sp. prefers isobutyric, butyric and propionic acids as initiators of fatty acid biosynthesis. The relative amount of the produced fatty acids is significantly influenced by the availability of intermediates or final products from the amino acid catabolic pathways. Antibiotic complex composition closely reflects the cell fatty acid pattern, in agreement with the assumption that the antibiotic fatty acid moieties are synthesized by shortening the chain of cell fatty acids.

Keywords Glycopeptide antibiotics · Cell fatty acids · Actinomycetes · Fermentations · Branched amino acids

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Introduction

Our interest in fatty acid metabolism in *Nonomuraea* ATTC 39727 derives from the observation of the close structural relationship between cell fatty acids and the shorter fatty acid moieties, which characterize the single components in the antibiotic complex produced by the strain. *Nonomuraea* cultures produce antibiotic A40926 [6], which is the starting material for the synthesis of dalbavancin, a clinically effective antibacterial antibiotic [5]. A40926 is a complex comprised of several structurally related factors (Fig. 1). Complex components are known to differ in the structure of the fatty acid moiety (R1) linked to the glucuronic acid residue of the molecule [19, 20]. Main components of the complex are A_0 (R1 = *iso*-C11:0), A_1 (R1 = *n*-C11:0), B_0 (R1 = *iso*-C12:0) and B_1 (R1 = *n*-C12:0). The origin of the fatty acids constituting the acyl moieties of the glycopeptide molecules has been previously studied in the structurally closely related teicoplanin [2], A40926 [20] and in the lipopeptide antibiotic ramoplanin [3]. Borghi et al. [2] studied factors affecting the normal and branched-chain acyl moieties of teicoplanin, and demostrated that teicoplanin acyl moieties are biosynthesized from longer cell fatty acids by shortening of the carbon chains. Since apparently the shortening is performed by the classical β-oxidation mechanism by loss of acetic acid units starting from the carboxyl end, the characteristic end terminal features (branched or linear) are conserved. More-

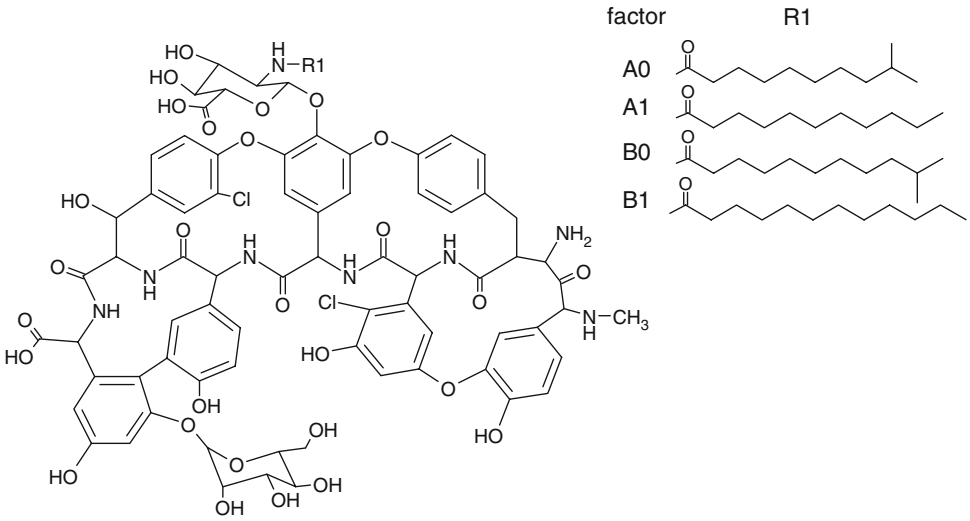

Fig 1 A40926 chemical structure. Complex components differ in the acyl moieties substituted in the position *R1* of the molecule

over, the characteristic even or odd number of carbons is also conserved. The hypothesis was confirmed by the biosynthesis of novel teicoplanins bearing ten carbon chains with structural variations by adding to the culture medium C18 acids having corresponding structural features [12].

The biosynthesis of linear and branched fatty acids in prokaryotes has been elucidated in detail [10]. Branched chain fatty acids are synthesized using as chain initiator the coenzyme A esters of isobutyric acid (resulting in fatty acid with an even number of C atoms), isovaleric acid (resulting in fatty acid with an odd number of carbon atoms) or 2-methyl butyric acid (resulting in *ante-iso* fatty acid with an odd number of C atoms). These initiators can be easily provided by reactions, common to all branched amino acids catabolic pathways, consisting of the conversion by valine dehydrogenase of the amino acids into the corresponding α-ketoacids, namely α-ketoisovaleric, α-ketoisocapronic and 3-methyl-α-ketovaleric acids followed by oxidative decarboxylation. However, other ways of synthesizing the primers are known; we may note that all the above α-ketoacids are produced as intermediates in the pathway of branched amino acid synthesis, and that in *Streptomyces* sp. isobutyric acid can by formed by isomerisation of *n*-butyric acid [8]. The biosynthesis of branched chain and straight chain fatty acids in actinomycetes has been studied [17, 18] by adding perdeuterated precursors to cultures grown on an amino acid rich medium. The nature and the origin of the chain initiators was demonstrated the same as that of bacilli. In the present contribution, we examine the biosynthesis of fatty acids in *Nonomuraea* cultures grown in a chemically defined minimal medium containing ammonium ions as the only nitrogen source. In particular, we assess the capacity of the strain of synthesizing the starters for the fatty acid chains, and the effect of biosynthetic precursors on both cell fatty acid composition and A40926 complex composition in the absence of potentially interfering nutrients usually present in rich media.

Materials and methods

Strains and cultural conditions

Nonomuraea sp. ATTC 39727 was maintained as a frozen vegetative stock at −80 °C in 15% glycerol at a biomass concentration of approximately 0.08 g/ml DW (dry weight). Fermentation in the chemically defined minimal medium P150 [7] was carried out in shake-flasks. For vegetative seed culture preparations, a 500-ml baffled Erlenmeyer flask containing 100 ml seed medium described in [1] was inoculated with one glycerol stock vial and incubated for 96 h at 28 °C on a rotary shaker (200 rpm). Subsequently, seed culture was used for inoculating (at 10% v/v) 100 ml aliquots of medium P150, and medium P150 supplemented with either 0.5 g/l of valine, 0.5 g/l of L-leucine or with 0.5 g/l of L-isoleucine, in 500-ml baffled Erlenmeyer flask. The shake-flasks were incubated at temperature of 28 °C on a rotary shaker (200 rpm) and fermentation monitored for 96 h.

Analysis

Fermentation broth samples were collected at regular time intervals and analyzed. Antibiotic production was monitored by HPLC analysis, as described in [1]. Biomass concentrations were determined by means of dry weight measurements. Glucose was analyzed using Trinder assay (SIGMA Diagnostics, St. Louis, MO, USA). Inorganic phosphate was determined by malachite green method [4]. The indophenol direct method was used to assay ammonium concentrations [9]. After 72 h of fermentation, composition of the cell fatty acids was determined as fatty acid methyl esters derivatives (FAMEs) by GC–MS method, previously described in [1].

Results and discussion

Growth and production in minimal medium

The fermentation parameters of *Nonomuraea* sp. grown in the chemically defined P150 medium without amino acid supplements (i.e., reference conditions) are given in Fig. 2. Data are mean values from three replicas of fermentations and each measurement was independently repeated three times.

In contrast to what is commonly known for many secondary metabolites, the production of the A40926 antibiotic was found to be partly associated with the cell growth. This pattern was consistent with batch fermentation data previously reported by Gunnarsson et al. [7]. Maximum antibiotic production of 16 mg/l was observed after 72 h of growth and it coincided with glucose exhaustion and concomitant end of biomass growth. At this culture time, samples of mycelium were collected and the composition of cell fatty acids was determined by GS–MS of their methyl esters (FAME).

To study the effect of branched amino acids, the fermentation was repeated by adding to the medium 0.5 g/l of L-valine, L-leucine or L-isoleucine, respectively, and the composition of the antibiotic complex as well as of the whole cell fatty acids was determined. Using relative abundance values, the percent contribution of the various FAMEs to the fatty acid composition was calculated and given in Table 1.

Cell fatty acid composition in minimal medium

In cells grown in medium P150 without supplements, linear acids with an even number of carbons predominated

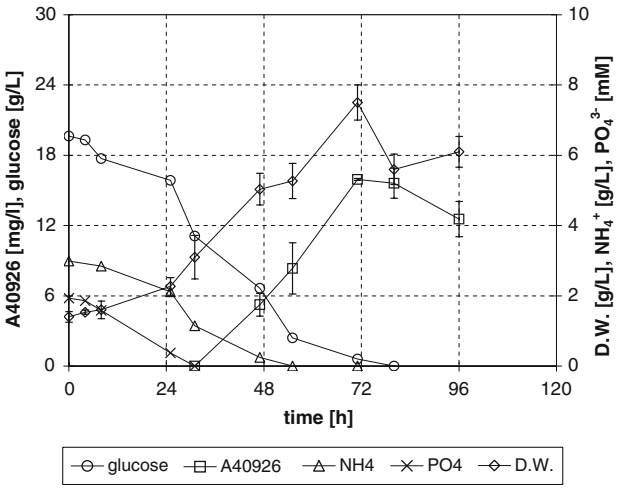

Fig 2 Fermentation time course of *Nonomuraea* sp. grown in chemically defined P150 medium (*error bars* give a 95% confidence region)

Table 1 Composition of cell fatty acids found under different fermentation and analyzed as FAMEs in GC–MS

Rt (min)	Fatty acid	Area (%)			
		P150	P150 + Val	P150 + Leu	P150 + Ile
13.94	*i* 14:0	–	0.64	0.61	0.56
14.96	*n* 14:0	4.44	2.76	2.78	1.82
16.65	*i* 15:0	1.16	3.52	5.02	2.69
17.61	*n* 15:0	1.68	6.54	1.35	10.33
18.63	*n* 14:0 3-OH	0.65	2.40	3.41	2.36
19.23	*i* 16:0	8.18	26.30	36.37	17.41
19.58	16:1.9	6.30	0.80	0.46	0.44
20.17	*n* 16:0	27.97	11.96	10.21	13.66
20.5	*n* 15:0 3-OH	–	–	17.38	1.23
21.25	*n* 16:0 10 met	13.16	1.87	3.33	1.28
21.72	*i* 17:0	0.78	0.76	1.78	–
21.96	*n* 17:1.9	1.21	3.36	0.99	4.59
22.62	*n* 17:0	1.20	4.87	1.05	6.41
23.59	*n* 17:0 10 met	8.40	23.98	8.02	32.21
24.28	*n* 18:1.9	2.92	0.91	0.40	–
24.73	*n* 17:0 3-OH	–	–	–	1.52
24.97	*n* 18:0	2.53	4.81	2.98	1.99
25.86	*n* 18:0 10 met	16.96	1.72	3.23	1.24
27.21	*n* 19:0	1.06	0.82	0.22	–
29.39	*n* 20:0	1.39	1.97	0.40	0.25

(Fig. 3a and Table 1). The major component was *n*-C16:0. Summing up the contribution of the two linear hexadecanoic acids (*n*-C16:0 and *n*-C16:1), it overcame the 35% of the total. This suggests a large availability of butyrate, previously identified as the primer of such hexadecanoic acids either in bacilli or in actinomycetes [17, 18]. Among the linear acids primed by propionate, 10-methylheptadecanoic acid predominated (8.40%). This is not surprising since according to [11, 21], the presence of this acid is a typical taxonomical feature of *Nonomuraea* genus. Acids, whose synthesis is initiated by isobutyrate (*iso*-C16:0), contributed with the 8.18% to the total, and those initiated by isovalerate contributed with merely the 2.46%. Given the apparent large availability of butyrate, we can surmise that in our cells isobutyrate derived mainly from the butyrate isomerisation. Indeed, a pathway of butyrate synthesis from acetate and its conversion to isobutyrate was previously demonstrated in *Streptomyces* sp. by several studies ([8] and the literature therein quoted). In agreement with the fatty acid composition typical for *Nonomuraea* genus, *anteiso*-acids were not detected (Fig. 3a, Table 1).

This fatty acid composition of *Nonomuraea* cells grown in minimal medium, substantially differs from that reported by [20] for *Nonomuraea* cells grown in a complex medium. In that condition, the two predominant components were *iso*-hexadecanoic acid and 10-methyleptadecanoic acid.

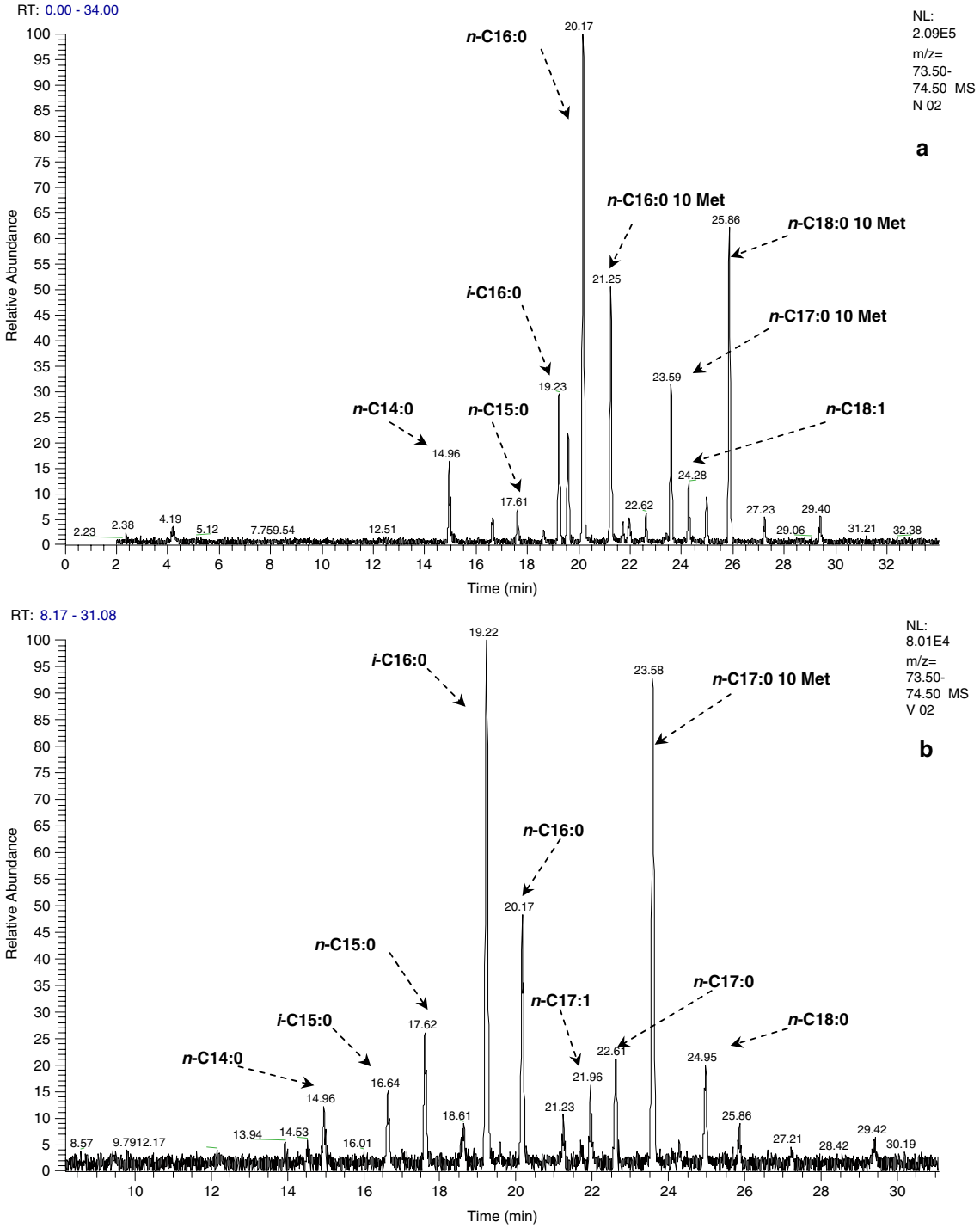

Fig 3 FAME GC–MS profiles for *Nonomuraea* sp. ATCC 39727 cell fatty acids from cultures grown in the chemically defined minimal medium P150 (**a**), and in medium P150 supplemented with 0.5 g/l of L-valine (**b**) or L-leucine (**c**) or L-isoleucine (**d**). Peaks relevant for the discussion are indicated

The first is primed by isobutyric acid, obviously provided by the partial catabolism of L-valine present in the culture medium. The second is primed by propionate, which is the end product of the catabolism of L-leucine and L-valine in many organisms [13] including streptomycetes [14, 16].

L-valine effect on cell fatty acid composition

In a previous paper from our laboratory, Beltrametti et al. [1] compared the pattern of fatty acid of cells grown in P150 medium with that of cells grown in P150 medium

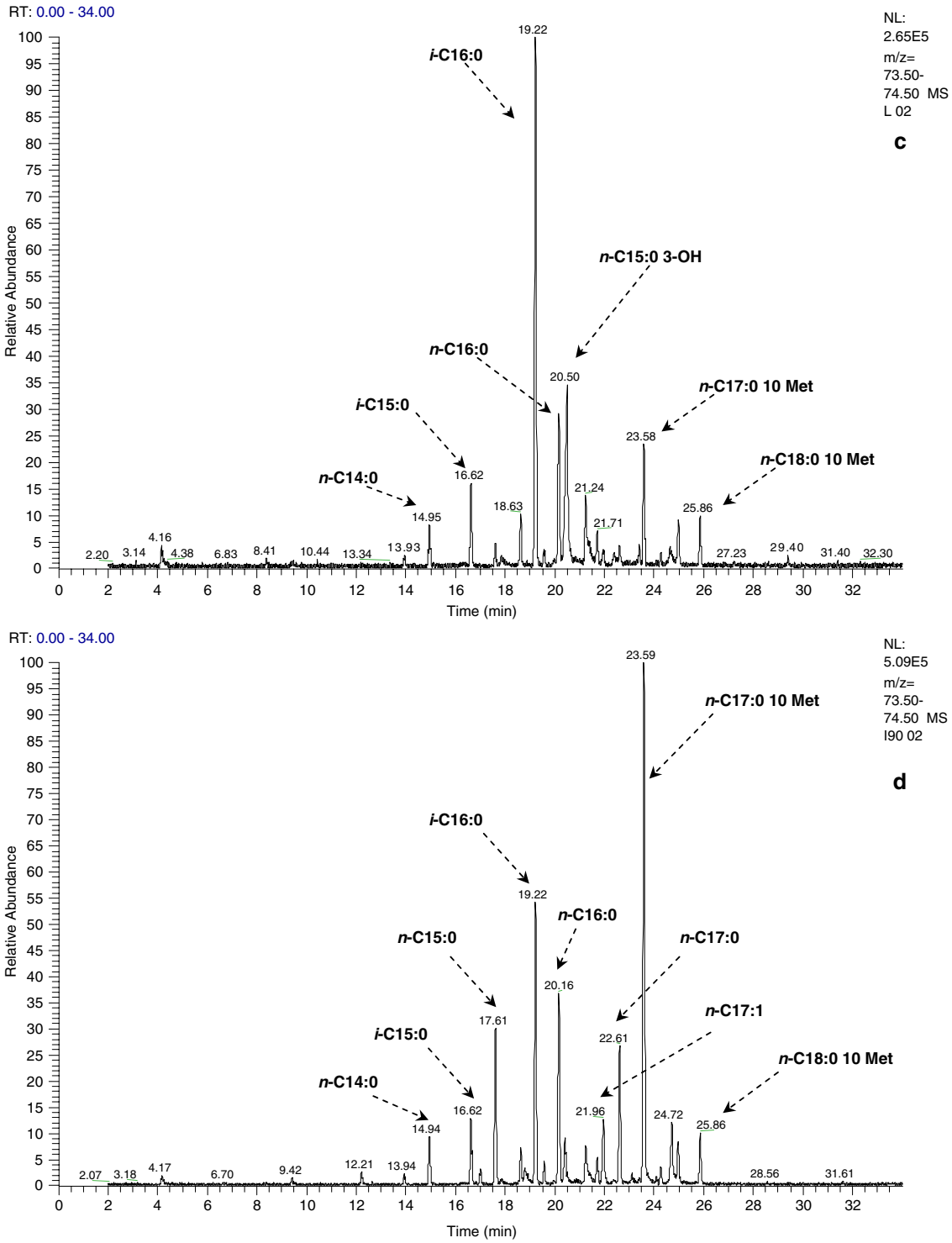

Fig 3 continued

plus 0.75 g/l of L-valine. Beside a positive effect on antibiotic titer (see below), L-valine addition resulted in a substantial increase of *iso*-C16:0 acid coupled with a significant decrease in the relative amount of linear *n*-C16:0 acid. The results hereby presented (Fig. 3b, Table 1) obtained with 0.5 g/l of L-valine revealed that *iso*-C16:0 was in fact predominant, representing the 26.30% of the total, whereas *n*-C16:0 contribution was decreased from about 30 to about 12%. Another major effect of L-valine appeared to be the increase of *n*-C17:0-10-methyl acid from 8.40 to 23,98%. The biosynthesis of this chain is initiated by propionate which is the end product of L-valine catabolism

pathway. Other relevant amounts were those of *n*-C17:0 and *n*-C15:0 acids (4.87 and 6.54%, respectively) also primed by propionate.

L-leucine effect on cell fatty acid composition

When P150 medium was supplemented with 0.5 g/l of L-leucine or L-isoleucine, no significant changes in the fermentation trend or in the total antibiotic production were observed (data not shown). However, upon addition of 0.5 g/l of L-leucine, important changes in cell fatty acid composition could be noticed (Fig. 3c, Table 1). The contribution of *iso*-fatty acids with an odd number of carbon (*iso*-C15:0 and *iso*-C17:0) increased to 6.80 from the 1.84% observed in the un-supplemented medium. This was indeed a relatively small contribution, indicating that isovalerate is a poor substrate for the fatty acid synthase and therefore is largely available to be converted into isobutyric acid, the end product of L-leucine catabolic pathway. This is consistent with the unpredicted large increase of *iso*-C16:0, which represented the 36,37% of the total. It may be added that a secondary way of formation of isobutyric acid is possible. In fact, in many bacteria, L-leucine in relatively low concentrations inhibits the activity of α-isopropylmalate synthase, which catalyzes the first step of leucine synthetic pathway, i.e., the conversion of α-ketoisovalerate into α-isopropylmalate. As a consequence, α-ketoisovalerate may accumulate and give rise, through oxidative decarboxylation, to isobutyric acid.

Considering the other relevant acid, it can be observed that the sum of the amounts of linear acids primed by propionate (*n*-C15:0, *n*-C17:0, *n*-C17:1, *n*-C17:0 10 Met), is approximately one-third of the amount of *iso*-C16:0.

L-isoleucine effect on cell fatty acid composition

Addition of L-isoleucine to the fermentation medium resulted in a striking increase of odd-carbon linear fatty acids, namely *n*-C15:0 (11.14%), *n*-C17:0 (6.91%), *n*-C17:1 (4.95%) and *n*-C17:0 10 met (34.35%) (Fig. 3d, Table 1). This was understandable considering that propionyl-CoA and acetyl-CoA are the end products from L-leucine catabolic pathway. The first could be directly used for odd-carbon acid initiation. Acetyl-CoA, according to the above-mentioned pathway, could produce butyrate or isobutyrate and contribute to the synthesis of the observed *iso*-C16:0 and *n*-C16:0. No *anteiso*- fatty acids were observed, in spite of the demonstrated role of L-isoleucine as enhancer of their synthesis in many organisms. This suggests that the 2-methylbutyrate produced by L-isoleucine degradation is not recognized as a suitable substrate for fatty acid initiation in *Nonomuraea* sp.

A40926 complex composition

As mentioned before, the acyl moieties in the A40926 complex are believed to originate from the degradation of cell fatty acids. Analysis of the A40926 complex produced in the described conditions, confirmed this assumption. The relative abundance of A40926 main factors is given in Fig. 4. In the fermentations carried out in medium P150 without supplements, the major antibiotic component was factor B_1, characterized by *n*-C12:0 acyl moiety. Its amount is 2.8-folds that of component B0 (*iso*-C12:0) and is 12.2- and 20-folds that of components A1 (*n*-C11:0) and A0 (*iso*-C11:0), respectively. There is a qualitatively good correspondence between A40926 complex and the cell fatty acid pattern, considering that the linear hexadecanoic acis predominated among fatty acids, followed in the order by the *iso*-hexadeanoic, the heptadecanoic acids and then by the acids primed by isovalerate.

Following the addition of L-valine (Fig. 4), the change in fatty acid composition was reflected by the change in the antibiotic complex composition. Component B0, characterized by an *iso*-C12:0 chain, became the major component of the antibiotic complex, in agreement with *iso*-C16:0 being the major component among cell fatty acids. The remarkable increase in *n*-C15:0 and *n*-C17:0 fatty acids was reflected by a major increase of A40926 factor A_1 (*n*-C11:0) synthesis. However, it appears evident that the 10-methyl-heptadecanoic acid, about as abundant as the i*so*-C16:0 acid, did not contribute to the formation of the *n*-C11:0 moiety of factor A1. In fact, by the loss of three acetate units, the acid would be converted into the 4-methyl-undecanoic acid, not accepted as a substrate by the transacylase. The small amount of factor A0 is consistent with the minor contribution of *iso*-C15:0 to the cell fatty acids.

In the case of L-leucine addition (Fig. 4), as could be predicted from the fatty acid composition, factor B0 was the major constituent of the antibiotic complex. In spite of a five times increase with respect to reference condition, the contribution of factor A_0 (characterized by an *iso*-C11:0 chain) was still inferior to that of factor B1 (with an *n*-C12:0 chain), consistently with the ratios of the fatty acids from which they respectively derive.

When L-isoleucine was added (Fig. 4), 10-methyl-hexadecanoic was the predominant cell fatty acid. As discussed above, this acid could not contribute to the formation of the antibiotic fatty acid moieties. All together the other acids initiated by propionate represented the 23% of the total, versus the 17.91% of those initiated by butyrate and the 17.41% of those initiated by isobutyrate. This distribution was reflected in the composition of the antibiotic complex in which factor A1 predominated, followed in the order by factors B1 and B0.

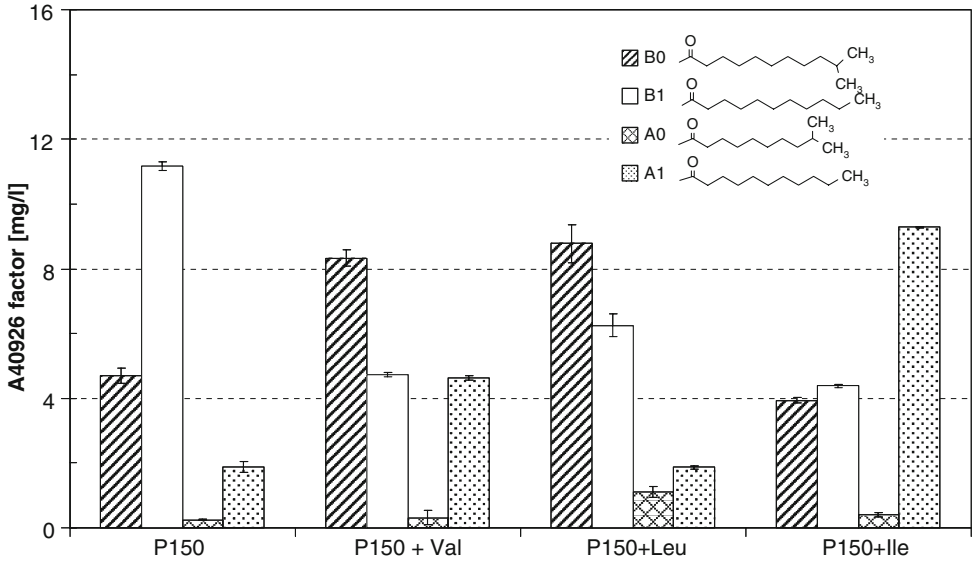

Fig 4 Composition of the A40926 complex in the chemically defined minimal medium P150 and in medium P150 supplemented with 0.5 g/l of L-valine or L-leucine or L-isoleucine (*error bars* give a 95% confidence region). The structures of the chains characterizing the factors are indicated

In conclusion, when a single branched amino acid was added to the fermentation medium, the biosynthesis of fatty acids was influenced not only by the products of the initial steps of its catabolism, but also by the end products of the pathway, which, as it is the case of propionate, acted as the starters of specific fatty acids. The overall result depended on a combination of the biosynthetic starters availability with the affinity of the starters for the synthase. It is evident that in *Nonomuraea* cells, three units, namely butyryl-CoA, isobutyryl-CoA and propionyl-CoA were about equally efficient in promoting the biosynthesis. Isovaleryl-CoA was accepted as a substrate to a lesser extent and 2-methylbutyryl-CoA was not recognized as a promoter. As a consequence in the case of L-isoleucine addition, no *anteiso* fatty acids were synthesized and the complete catabolism of the amino acid provided large amounts of propionyl-CoA, the primer of fatty acids with an even number of carbons. The case of L-leucine is also interesting since, due to the low affinity of isovalerate for the synthase, this intermediate was largely available for further degradation and was metabolized into isobutiryl-CoA, the end product of L-leucine catabolism. Therefore, the prominent cell fatty acid was the *iso*-hexadecanoate, rather than the expected *iso*-C15 or *iso*-C17 acids.

In all the conditions examined, either in the absence or in presence of different amino acids, the relative proportion of the components in the antibiotic complex was qualitatively corresponding to the distribution of cell fatty acids. This could hardly be the case if the antibiotic acidic moieties were synthesized ex novo by a different synthase, and therefore constitutes a further confirmation of the origin of these moieties as degradation products from longer chain fatty acids. Sequencing of A40926 biosynthetic gene cluster [15] revealed that genes devoted to fatty acid synthesis were not present in the indeed complete and highly organized cluster, further indicating that the acyl moieties introduced in the A40926 originate from cell fatty acid turnover.

This observation may be of practical value since it indicates the possibility of altering the composition of the A40926 complex by the addition of suitable precursors and of obtaining new derivatives by supplementing the fermentation medium with 16- or 18-carbons acids bearing desired structural features.

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