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Valine Influences Production and Complex Composition of Glycopeptide Antibiotic A40926 in Fermentations of *Nonomuraea* sp. ATCC 39727

FABRIZIO BELTRAMETTI*, SRDJAN JOVETIC, MARINA FEROGGIO, LUCIANO GASTALDO, ENRICO SELVA and FLAVIA MARINELLI

Vicuron Pharmaceuticals Italy S.r.l., Via R. Lepetit, 34, 21040, Gerenzano (Varese) Italy

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In actinomycetes the catabolism products of branched chain amino acids provide biosynthetic precursors for the formation of several lipid-containing antibiotics. We have determined in *Nonomuraea* sp. ATCC 39727 the effect of valine on production of glycopeptide antibiotic A40926, which is a complex of factors structurally differing in fatty acid moieties. Addition of valine to minimal medium increased A40926 production and modified complex composition towards a mono-component. Similar results were also obtained in a rich production medium.

The glycopeptide antibiotic complex A40926[1,2] is the natural precursor of the semi-synthetic derivative dalbavancin, which is currently under clinical development at Vicuron Pharmaceuticals. Dalbavancin shows excellent activity against staphylococci, streptococci and enterococci including some VanB isolates and has interesting pharmacokinetic characteristics[3]. A40926 core peptide possesses the typical heptapeptide structure of the D-alanyl-D-alanine binding glycopeptides of the teichomycin sub-group and it is characterized by a *N*-acylaminoglucuronic acid and a mannose moiety bound to the amino acids number 4 and 7 respectively (Figure 1). Several structural analogues have been identified as components of the A40926 complex[4,5]. The two major components of the complex, named B0 (formerly B) and B1, are characterized respectively by an *iso*-C12:0 and a *n*-C12:0 acyl moiety bound to the aminoglucuronic acid of the antibiotic (Figure 1). B0 and B1 are typically the most abundant components of the A40926 complex.

A40926 is produced by fermentations of the actinomycete *Nonomuraea* sp. ATCC 39727 (formerly *Actinomadura* sp. ATCC 39727)[4,6]. The addition of valine as potential precursor of the branched acyl chain of the B0-A40926 factor was investigated. The importance of this amino acid as branched chain fatty acid primer was evidenced in the biosynthesis of several antibiotics[7~9]. In particular, the effect of valine on complex composition and antibiotic production was evidenced for teichomycin glycopeptide, which is closely related to A40926[10]. The influence of valine on A40926 production was first evaluated in a chemically defined growth medium. The positive effect observed in minimal medium was also observed in a rich medium, potentially suitable for industrial fermentation[11,12].

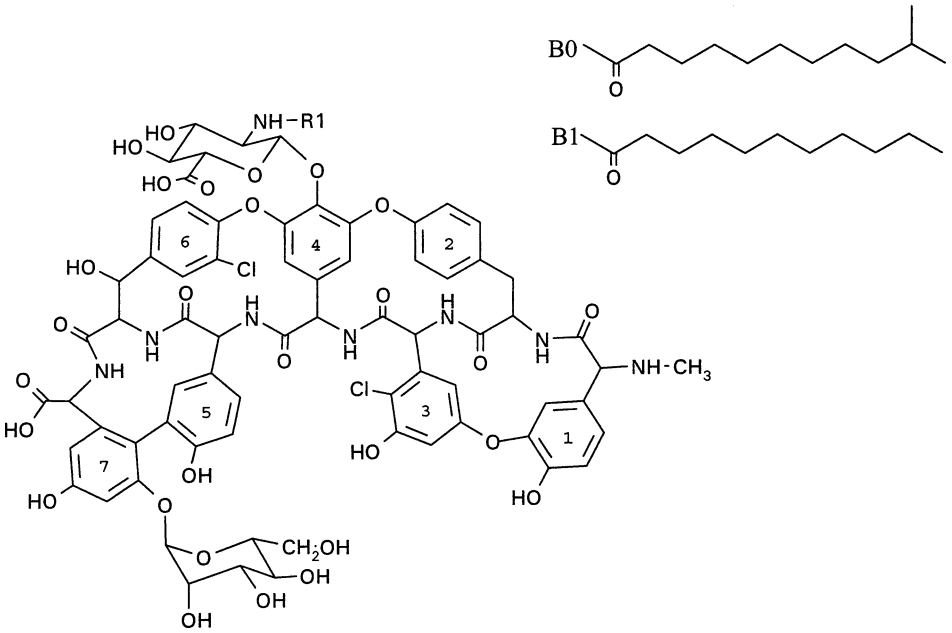
Materials and Methods

Strains and Cultural Conditions

Nonomuraea sp. ATTC 39727 was maintained as a lyophilised Master Cell Bank (MCB). A Working Cell Bank (WCB) of the strains was prepared from the first generation slant originating from the MCB inoculated in V6 medium (grams per litre: glucose 20, meat extract, 5; peptone, 5; yeast extract, 5; casein hydrolysate, 3; NaCl, 1.5; demineralised water up to 1 litre, pH 7.4 with NaOH), grown for 96 hours and stored in 1.5 ml cryo-vials at −80°C.

* Corresponding author: fbeltrametti@ricuron.it

Fig. 1. Structure of the A40926 major components B0 and B1.



R1 indicates the position of the alternative B0 or B1 acyl moieties.

Fermentation in T/2 medium was performed as described by BELTRAMETTI *et al.*[11]. In brief, cryo-vials of the WCB were thawed at room temperature and 2 ml were used to inoculate 100 ml of vegetative medium E25[12] in 500-ml baffled flasks. Strains were grown for 72～96 hours on a rotary shaker at 200 rpm and 28°C. Fermentation was started with a 10% inoculum from the vegetative medium flask in 100 ml of production medium T/2[12] modified according to SELVA *et al.*[2]. L-Valine was added from a 20% w/v stock (pH 7) to the fermentation flask at different concentrations and the fermentation was allowed for 168 hours at 28°C with shaking at 200 rpm.

Fermentation in minimal medium P150[13] was performed as follows. Cryo-vials of the WCB were thawed at room temperature and 2 ml were used to inoculate 100 ml of seed medium in 500-ml baffled flasks. Composition of seed medium was in grams per liter: glucose 45, $(NH_4)_2SO_4$ 8.3, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 1, $CaCO_3$ 5, yeast extract 1, deionised water up to, pH 7.5. 3 ml of TMS-1 solution (in grams per litre: $FeSO_4 \cdot 7H_2O$ 5, $CuSO_4 \cdot 5H_2O$ 0.39, $ZnSO_4 \cdot 7H_2O$ 0.44, $MnSO_4 \cdot H_2O$ 0.15, $NaMoO_4 \cdot 2H_2O$ 0.011, $CuCl_2 \cdot 2H_2O$ 0.020; HCl 37% 50 ml) were added to the medium above described to supply trace metals. Strains were grown for 96 hours on a rotary shaker at 200 rpm and 28°C. Fermentation was started with a 10% inoculum from the vegetative medium flask in production medium P150 with the addition of 1 ml/litre of vitamin solution 1 (in grams per litre: biotin 0.05, Ca-pantothenate 1, nicotinic acid 1, *myo*-inositol 25, thiamine-HCl 1, pyridoxine-HCl 1, *para*-aminobenzoic acid 0.2)[13]. L-Valine was added from a 20% w/v stock (pH 7) to the fermentation flask at different concentrations and the fermentation was followed for 120 hours at 28°C with shaking at 200 rpm.

A40926 Extraction and Analysis, Dry Weight, PMV and pH Determination

Samples were collected from fermentation flasks at different time intervals and processed for A40926 extraction as already described[11]. The whole bacterial culture was brought to pH 11 with NaOH and filtered through a 0.45 μm durapore membrane filter (Millipore). The filtrated broth was incubated at 50°C for 1 hour and then directly analysed by HPLC (see below). 10 ml of culture were collected from the fermentation flask, centrifuged at 3250×g for the determination of Packed Mycelium in Volume (PMV(%)) and pH. Alternatively, 10 ml of culture were brought to pH 4 with the addition of HCl 37% in order to dissolve $CaCO_3$, filtered through pre-dried 0.45 μm durapore membrane filters (Millipore) and dried at 80°C for approximately 24 hours. The filter was

then weighed and the dry cell weight (DCW) in grams per litre of fermentation culture determined. Growth rate was determined during the exponential growth phase as PMV(%) or DCW per litre per hour of growth (PMV(%)$\cdot h^{-1}$ or DCW$\cdot L^{-1}\cdot h^{-1}$). All the results herein reported are the mean of at least two independent experiments.

Analytical HPLC

HPLC analyses were performed as already described[11] on a 5 μm particle size Ultrasphere ODS (Beckman) column (4.6×250 mm) eluted at 1 ml/minute flow rate with a 26 minutes linear gradient from 25% to 37% of Phase B. Phase A was 20 mM $HCOONH_4$ pH 4.5 buffer : CH_3CN 95 : 5 (v/v) and Phase B was 20 mM $HCOONH_4$ pH 4.5 buffer : CH_3CN 5 : 95 (v/v) mixture. The chromatography was performed with a Hewlett Packard mod 1100 HPLC system and detection was at 254 nm. As internal standard an authentic sample of A40926 antibiotic was used. A40926 production was herein reported as the sum of the B0 and B1 components amount.

Analysis of Fatty Acids

Analysis was performed essentially as previously described[5,10]. In brief, mycelium was collected by centrifugation and 15 mg were saponified with 1 M NaOH in 3 ml of methanol/toluene (70 : 30 v/v) at 100°C for 30 minutes. Acidification with 3 ml of 2% HCl in methanol, incubation for 30 minutes at 85°C and addition of 1 ml of 14% (w/v) BF_3/methanol reagent followed by incubation for 10 minutes at 100°C (Supelco BF_3-methanol Kit 3-3020) allowed conversion of fatty acids to their methyl esters (FAMEs). FAMEs were extracted twice with 3 ml of hexane and the solvent was concentrated to 0.25 ml. Gas chromatography-mass spectrometry (GC-MS) analysis of FAMEs was carried out on a Thermo Finnigan Trace GC-MS instrument, equipped with PTV injector, operating in electron impact ionization mode. 1 μl of hexane solution was injected into a Restek RTX-5MS fused silica capillary column (internal diameter 0.25 mm, length 15 metres, film thickness 0.25 μm).

The following method was used. Temperature programme: 4 minutes at 100°C, from 100 to 250°C at 4°C min^{-1}, 5 minutes at 250°C. Injector: splitless mode, base temperature 50°C, transfer temperature 250°C, transfer rate 14.5°C$\cdot min^{-1}$.

Results and Discussion

Fermentation of *Nonomuraea* sp. ATCC 39727 in Minimal Medium

The catabolism of branched chain amino acids in actinomycetes proceeds *via* intermediates that are precursors to a wide range of antibiotics[14]. Typically, valine dehydrogenase (VDH) is responsible for the oxidative deamination of all the branched chain amino acids. The second enzyme in the branched chain amino acid

Table 1. Influence of valine on growth in minimal P150 medium.

Valine (g/L)	[a]Growth rate (g of DCW·L^{-1}·h^{-1})	[b]g of DCW·L^{-1} at harvesting
0	0.030	5.4 (+/-0.0)
0.25	0.042	6.2 (+/-0.4)
0.5	0.052	6.8 (+/-0.0)
0.75	0.045	6.3 (+/-0.4)
1	0.052	6.4 (+/-0.0)
1.5	0.052	6.4 (+/-0.0)
2	0.052	6.2 (+/-0.3)
2.5	0.046	6.2 (+/-0.1)
3	0.040	5.8 (+/-0.0)

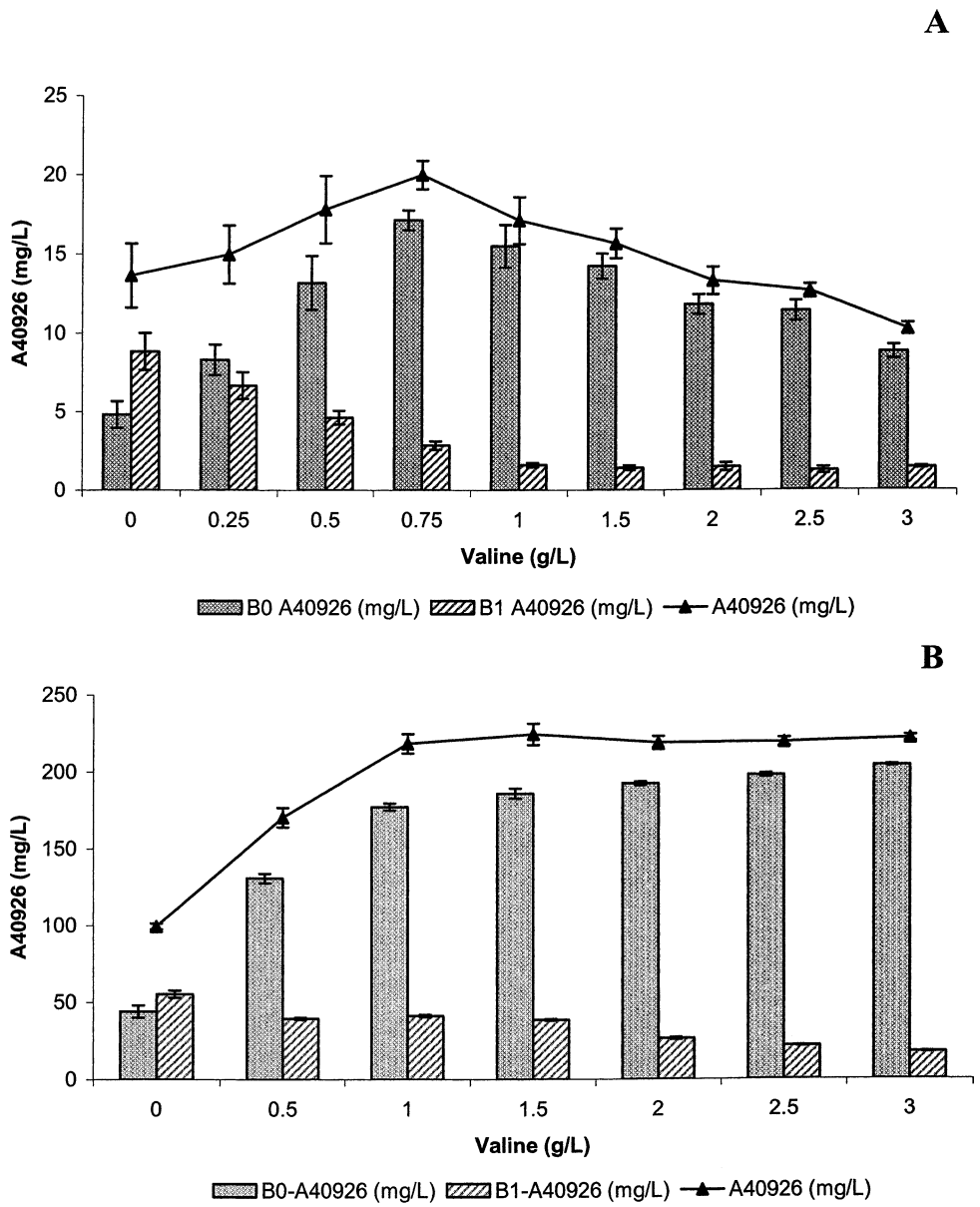
[a] Growth rate was determined during the exponential growth phase

[b] Harvesting was performed at the maximum level of biomass production (96-120 hours).

The value of 1 Standard Deviation is indicated in brackets

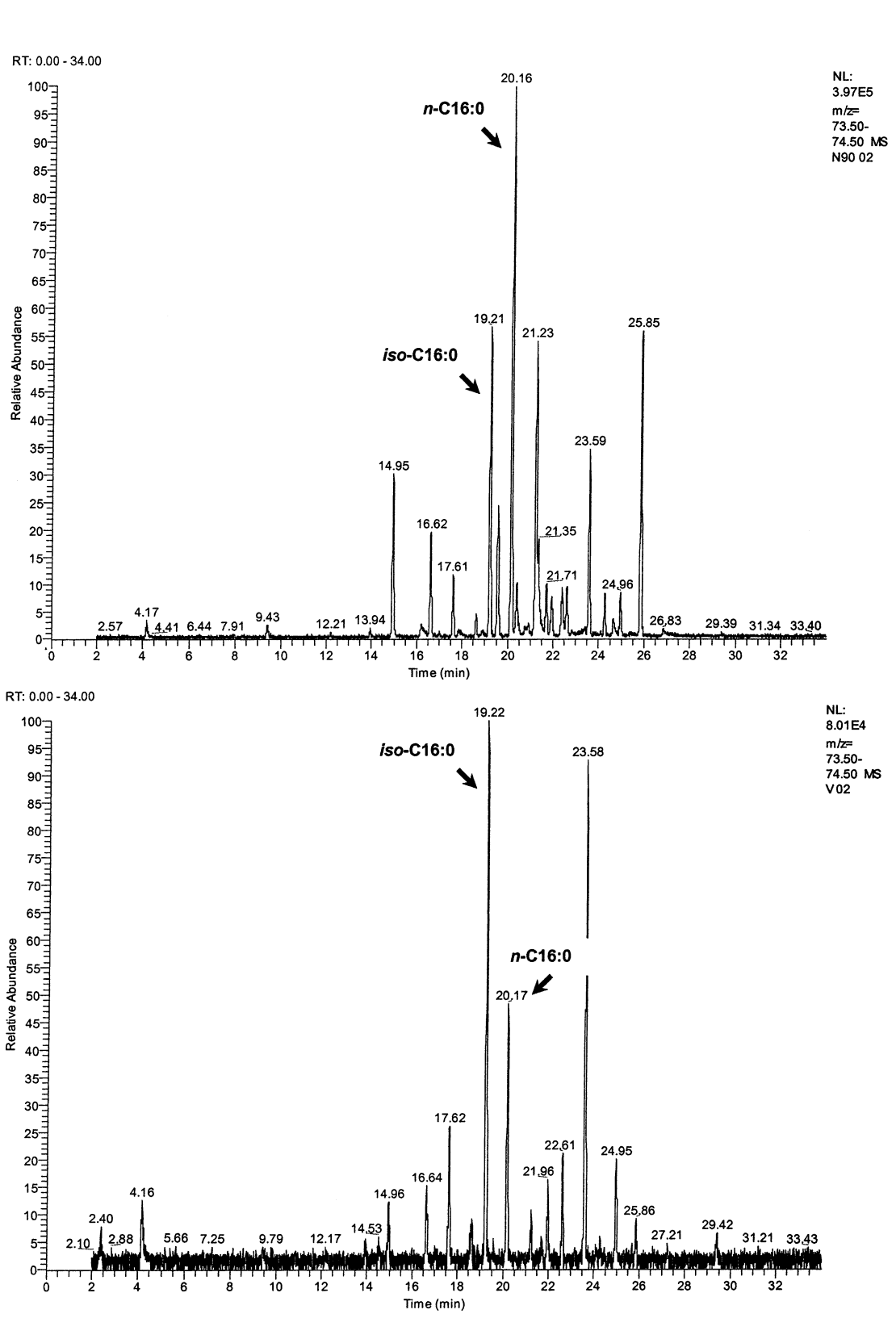
catabolism pathways is an α-keto acid dehydrogenase responsible for the conversion of α-ketoisovalerate (the product of VDH action on valine) into *iso*-butyrate[14] which is the starting molecule in the polymerisation process leading to even-carbon *iso*-acids[15]. To assess the influence of valine on A40926 production by *Nonomuraea* sp. ATCC 39727, we have performed fermentations in minimal P150 medium. The results suggest that valine exerts a positive effect both on growth and on production of the A40926 complex component, which has the even carbon *iso*-acid consistent with a valine-derived precursor. In fact, the addition of valine to medium P150 gave a consistent improvement in growth rate and DCW at harvesting (Table 1). Moreover, addition of valine increased both the relative and absolute production of the B0 component (characterized by an *iso*-C12:0 moiety) in the A40926 complex and indeed decreased the B1 factor (characterized by an *n*-C12:0 moiety) (Figure 2A). The relative amount of the two components was changed progressively from 1 : 2 ratio in the absence of valine (the control) up to the

Fig. 2. Effect of different valine concentrations on A40926 production by *Nonomuraea* sp. ATCC 39727.



Lines show maximum A40926 production in medium P150 (A) and T/2 (B) following the addition of increasing valine concentrations. Major A40926 species B0 and B1 are reported as bars in the corresponding experimental condition. The value of +/− 1 standard deviation is indicated in graph.

Fig. 3. FAME GC-MS profile for *Nonomuraea* sp. ATCC 39727 whole cell hydrolysates from cultures grown in medium P150 (A) and P150 supplemented with valine 0.75 g/liter (B).



Relevant peaks are: 13.94 minutes, *iso*-C14:0; 14.96 minutes, *n*-C14:0; 16.63 minutes, *iso*-C15:0; 17.62 minutes, *n*-15:0; 19.22 minutes, *iso*-C16:0; 20.17 minutes, *n*-C16:0; 21.7 minutes, *iso*-C17:0; 22.62 minutes, *n*-C17:00; 23.58 minutes, *iso*-C18:0; 24.96 minutes, *n*-C18:0; 25.85 minutes, C18:0 10 methyl.

maximum of 9 : 1 when 1.5~3 g/litre valine were added. The maximum in total A40926 production (146% increase, in respect to the control) was reached when valine was added at 0.75 g/litre (Figure 2A). Higher concentrations of valine gradually decreased total A40926 production to the control values (14 mg/litre).

GUNNARSON *et al.*[13] observed in fermentations of *Nonomuraea* sp. ATCC39727, that high initial ammonium concentrations resulted in impaired growth and decreased A40926 production, while ammonium limitation caused a stop in the antibiotic production. Thus, the positive effect observed on growth may be due to the presence of valine as a slow release nitrogen source. In the valine concentration range 0.75 g/litre~3 g/litre, growth was not impaired but A40926 production decreased with the maximum achieved with valine 0.75 g/litre. Production was repressed at high valine concentrations, possibly because of excess nitrogen deriving by deamination. A similar effect was observed in spiramycin production by *Streptomyces ambofaciens* (A23). In this case valine induced VDH and antibiotic production whereas ammonium suppressed VDH activity and antibiotic formation[16~18].

The valine effect in increasing component B0 yields is consistent with its conversion into *iso*-butyric acid (the precursor of branched even carbon fatty acids). This precursor may theoretically be used for the direct bio-synthesis of *iso*-dodecanoic acid, and therefore of component B0. However, DNA sequencing of the A40926 biosynthetic cluster did not evidence genes devoted to fatty acid synthesis[20]. In addition, it has been demonstrated that in the teichomycin complex, closely related to the A40926 complex, the acyl chains of the components are not synthesised directly, but derive from the shortening (through the classical β-oxidation pathway) of membrane fatty acids or of exogenous C_{18} acids fed in the fermentation medium[10,19]. Accordingly, valine may influence the composition of the pool of membrane fatty acids that ultimately are the precursors of the A40926 components, as observed for teichomycin. To evaluate this hypothesis we performed the analysis of membrane fatty acids (as FAME derivatives) extracted from *Nonomuraea* sp. ATCC 39727 grown on medium P150 with and without the addition of 0.75 g/litre of valine. The results show the presence of major peaks of linear even-carbon-acids in medium P150, whereas the addition of valine determines the relative increase in branched *iso*-C16:0 and *iso*-C18:0 acids (Figure 3). The ratio between *iso*-C16:0 and *iso*-C18:0 and the corresponding *n*-acids was 0.29 and 3.32 in medium P150 and 2.2 and 4.98 in medium P150 added with 0.75 g/litre valine respectively (Table 2). The marked increase in *iso*-C16:0 acid paralleled the increase in B0 component of the A40926 complex and the decrease of the B1 factor in the same condition, in agreement with the hypothesis of antibiotic fatty acids deriving from the membrane fatty acids.

Fermentation of *Nonomuraea* sp. ATCC 39727 in Rich Medium

Although a minimal medium is most suitable for physiology studies, higher production of antibiotic is usually obtained in rich media. To develop a convenient process we have added valine concentrations increasing up to 3 g/litre to fermentation medium T/2. Samples were analysed for A40926 production and antibiotic complex composition. Growth was approximately estimated as Packed Mycelium Volume referred to 100 ml culture (PMV%). Medium T/2 contains indeed about 5% amount

Table 2. Ratio between *iso* and *n* methyl fatty acids (FAMEs) of *Nonomuraea* sp. ATCC 39727 grown in medium P150 with and without valine 0.75 g/liter (GC-MS analysis)[a].

FAMEs	Ratio *iso*/*n* in medium P150	Ratio *iso*/*n* in medium P150 supplemented with 0.75 g/L valine
n-C14:0 / *iso*-C14:0	[b]ND	0.23
iso-C15:0 / *n*-15:0	0.69	0.54
iso-C16:0 / *n*-C16:0	0.29	2.2
iso-C17:0 / *n*-C17:00	0.65	0.16
iso-C18:0 / *n*-C18:0	3.32	4.98

[a] FAMEs were analyzed after 90 hours growth

[b] Not determined

Table 3. Influence of valine on growth in T/2 production medium.

Valine (g/L)	[a]Growth rate (PMV(%)·h^{-1})	[b]PMV(%) at harvesting
0	0.146	34.7 (+/- 1.2)
0.5	0.111	32.7 (+/- 0.6)
1	0.049	29.3 (+/- 0.6)
1.5	0.042	27.7 (+/- 0.6)
2	0.049	26.7 (+/- 0.6)
2.5	0.049	26.7 (+/- 1.2)
3	0.063	27.0 (+/- 1.0)

[a] Growth rate was determined during the exponential growth phase

[b] Harvesting was performed at the maximum level of biomass production (168-216 hours).

The value of 1 Standard Deviation is indicated in brackets

of insoluble components that influence DCW determination. However, values at harvest (27~36%) were only marginally affected, considering also that the medium insoluble material was partially or completely consumed to sustain the biomass production.

The addition of valine increased both the relative and absolute production of the B0 component in the A40926 complex and decreased the B1 factor, as observed for medium P150 (Figure 2B). The relative B0 : B1 composition changed progressively from an initial 1 : 1 ratio in the control experiment up to 10 : 1 when valine was added at 3 g/litre. Total production was 220%, in respect to the control, at valine concentrations ranging from 1 g/litre to 3 g/litre. In contrast to what was observed in minimal medium, A40926 production remained constant following the addition of valine in the range from 1 to 3 g/litre concentrations, although growth was generally negatively influenced as shown in Table 3.

Conclusions

We demonstrated that in fermentations of *Nonomuraea* sp. ATCC 39727 addition of valine determined both an increase in the relative amount of the B0-A40926 component and a modification of membrane fatty acids consistent with the observed changes in complex composition. Valine showed positive effect also in total complex production. This was observed both in minimal medium and in rich medium. Rich media typically sustain higher antibiotic productivities. However, it is rather common that medium modifications found positive in improving the relatively low production in minimal media do not result in any positive effect in industrial media. In the case of *Nonomuraea* sp. ATCC 39727, the A40926 valine-dependent increase in yield in minimal medium (from 14 to 20 mg/litre) was maintained in rich industrial medium (from 100 to 220 mg/litre).

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