Journal of Industrial Microbiology, 6 (1990) 279–284
Elsevier

SIM 00293

Bioconversion of avermectin into 27-OH avermectin

M. Chartrain, R. White, R. Goegelman, K. Gbewonyo and R. Greasham

Merck and Company, Inc., Rahway, NJ, U.S.A.

(Received 20 October 1989; revised 4 January 1990; accepted 8 January 1990)

Key words: Fermentation; *Nocardia*; Bioconversion; Avermectin

SUMMARY

The bioconversion of avermectin to its 27-hydroxy derivative is achieved with *Nocardia autotrophica* subsp. *canberrica*. The approach of increasing bioconversion productivity rather than efficiency was adopted in these studies. Process improvement studies focused on the physico-chemical conditions of the fermentation, examined initially at the shake-flask scale. Bioconversion yields were affected by pH, substrate concentration, time of substrate addition, substrate solubilization, carbon to nitrogen ratio, and medium strength. Optimization of these parameters resulted in a 8-fold process improvement. During pre scale-up studies, the sensitivity of this bioconversion to the antifoam employed was demonstrated and lard oil was selected as giving the best results. Additional process changes were required during scale-up efforts in larger vessels, including replacement of the original substrate solvent with dimethylsulfoxide.

INTRODUCTION

Microbial bioconversions are widely used in the chemical and pharmaceutical industry to perform site directed and stereo specific modifications of organic molecules [5,10]. The microbial biotransformation of drugs (i.e., steroid, antibiotics, vitamins, etc.), and fragrances are performed on a routine basis in these industries [7–9]. Bioconversions are of economical importance as they allow the synthesis of optically pure molecules at relatively low cost [15].

Avermectin, a secondary metabolite of *Streptomyces avermitilis* is chemically converted to ivermectin [12], an extremely powerful antihelmintic drug with a broad spectrum [1,3]. It is used worldwide for the treatment and prevention of nematode infections in cattle, horses, dogs, and even the treatment of infected wild animals has been reported (Taylor, W.P., Jr. and T.H. Spraker, Proceedings — 1983 Annual Meeting, American Association of Zoo Veterinarians, Tampa, FL, October 24–27, 1983 and Seward, R.L. and E.S. Brokken. Proceedings of the Heartworm Symposium 1986, New Orleans, LA, March 21–23, 1986. American Heartworm Society 1986, 1–8 (43)). More recently it was successfully used for the prevention of onchocerciasis (river blindness) in humans in Africa [17]. The microbial bioconversion of avermectin to 27-OH avermectin by *Nocardia autotrophica* subsp. *canberrica* was recently reported (Goegelman, R.T., E. Inamine and R.F. White, Eur. Pat. Appl. EP 212,867 and U.S.A. Pat. Appl. 20675/1042A). The hydroxylation of the molecule at the carbon number 27 position [16] (see Fig. 1) gives a very reactive site for subsequent site-directed chemical modifications of the molecule.

The goals of the present studies were first to optimize the production of 27-OH avermectin in shake flasks and second to scale up the process to small and medium size fermentors (14 and 800 liters, respectively) in order to produce large quantities of 27-OH avermectin.

MATERIALS AND METHODS

Chemicals. Peptone, yeast extract and beef extract were purchased from Difco (Detroit, MI). Other chemicals used in shake flask experiments were obtained from Fisher Scientific (Springfield, NJ). The antifoams used were obtained from the following vendors: FD 62 (Hodag, Skokie, IL); Pluronic L 122, Tetronic 901, and Pluracol P 2010 (BASF, Parsippany, NJ); Proflo oil (Traders, Memphis, TN); Lard burning oil (PFAU, Jeffersonville, IN). Avermectin Bla was obtained from Merck and Co., Rahway, NJ [16] and will be referred to in this article as avermectin.

Bioconversion. Nocardia autotrophica subsp. *canberrica* MA. 6181 (ATCC, 35203) was the strain used in these studies [13]. Inoculum development and avermectin bioconversion media were identical and contained per liter of distilled water: glucose, 10 g; beef extract, 1 g; yeast extract, 2 g; and peptone, 3 g. The pH was adjusted to 7.0 prior to autoclaving for 20 min at 121 °C.

Correspondence: M. Chartrain, Merck & Co., Inc., Building 80Y-105, P.O. Box 2000, Rahway, NJ 07065, U.S.A.

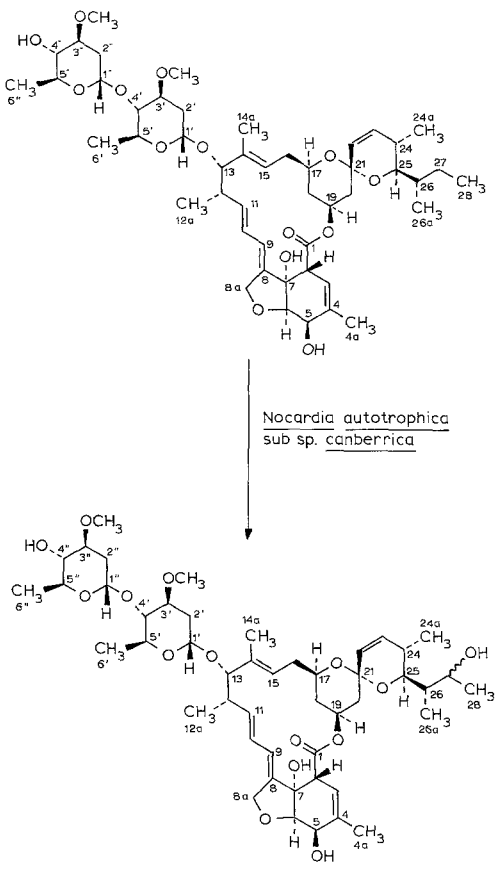

Fig. 1. Bioconversion of avermectin into 27-OH avermectin catalyzed by *Nocardia autotrophica* subsp. *canberrica*.

A frozen cell suspension (1 ml) was used to inoculate a 250 ml Erlenmeyer flask containing 50 ml of medium. The culture was incubated at 28 °C with shaking (220 rpm) for 48 h. The subsequent stage seeds were prepared using a 5% inoculum and used the same incubation conditions.

The bioconversion experiments were performed in 250-ml or 2-l Erlenmeyer flasks that contained either 20 ml or 500 ml of medium (metal closure flasks, Bellco Vineland, NJ). The flasks were inoculated with 5% inoculum. Immediately after inoculation, a methanol solution (0.25 ml) containing 500 000 units/l of avermectin was added. The final concentrations of avermectin and methanol were 6250 units/l and 1.25%, respectively. The cultures were incubated for 10 days in the previously described conditions. The optimized medium contained: glucose, 20 g/l; beef extract, 3 g/l; yeast extract, 6 g/l and peptone, 9 g/l. The pH of the optimized medium was adjusted to 4.0 prior to autoclaving.

Analytical techniques. Avermectin and 27-OH avermectin were extracted by pooling 20 ml of whole broth with 20 ml of methylene chloride into a 50 ml polyethylene centrifuge tube. The tubes were shaken for 20 min and the emulsion was broken by centrifugation at 2500 rpm for 20 min. The methylene chloride layer was recovered, taken to dryness and the residue solubilized in 3 ml of methanol. The methanol fraction was filtered through a 0.45 μm membrane and analyzed for avermectin and 27-OH avermectin. A high pressure liquid chromatograph (model 8800 DuPont Instruments, Wilmington, DE), equipped with an autosampler (model 725, Micromeritics, Norcross, GA), a UV detector (Spectromonitor III, Laboratory Data Control, Riviera Beach, FL) and a computing integrator (model 4100, Spectra Physics, San Jose, CA) was used. The sample (10 μl) was injected into a Zorbax ODS column (0.46 cm × 25 cm) (DuPont Instruments) operated at 60 °C. The solvent system was 85/15 (v/v) methanol/water at a flow rate of 1 ml/min. The eluant was monitored at 243 nm 0.1 a.u.f.s. The retention times of avermectin and 27-OH avermectin were 13.5 min and 7.2 min, respectively. The broth titers of 27-OH-avermectin-Bla were calculated, using an avermectin-Bla standard to yield a calculation factor equal to area count/unit, uncorrected for difference in molecular weight.

Scaled-up fermentations. Small scale fermentations were performed in 14-liter fermentors. The vessels contained 10 l of medium and were batch sterilized in an autoclave for 60 min at 121 °C. A volume of 500 ml of a second stage seed was used to inoculate the fermentor. The fermentor was operated at 28 °C, 400 rpm with an aeration rate of 4 l of air per minute. Aeration was reduced to 1 l per min after 48 h in order to minimize foam formation.

Medium size fermentors (800 l) contained 475 l of medium and were batch sterilized at 121 °C for 25 min. A volume of 25 l of a third seed stage was used to inoculate the fermentor. The fermentors were operated at 28 °C, 150 rpm with an aeration rate of 50 l of air per min. The back pressure in the fermentors was maintained at 0.3 kg/cm^2. The exhaust air from the fermentors was passed through an activated charcoal absorbant column to retain DMSO vapors before venting to the atmosphere.

RESULTS

Bioconversion kinetics

Fig. 2 presents a typical growth and avermectin bioconversion time course by *Nocardia autotrophica* MA 6181 in a 2-l shake flask. Glucose was consumed during the first 5 days of the fermentation with most of the biomass produced during the first three days. The pH of the fermentation medium stayed between 6.8 and 7.5 during the incubation period. Avermectin was added at inoculation and 27-OH avermectin production was detected

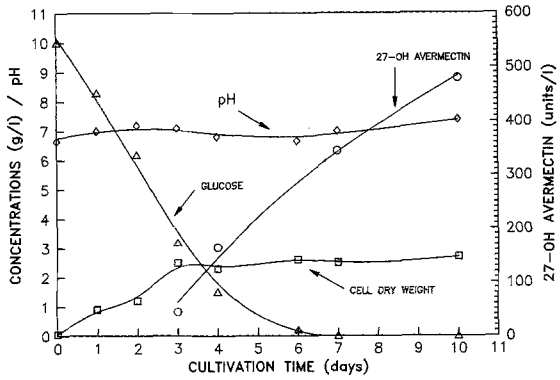

Fig. 2. Time course of avermectin bioconversion to 27-OH avermectin by *Nocardia autotrophica* conducted in a 2-l shake flask containing 500 ml of medium.

after three days and continued until day 10. No significant additional bioconversion was detected after that time. The maximum concentration of 27-OH avermectin reached was 420 units/l.

Effect of pH

The effect of the medium pre-sterile pH value was investigated over a pH range of 2.5 to 8.0. Very poor growth was observed in media with initial pH values lower than 3.5. A constant increase in the final concentration of 27-OH avermectin at 10 days was noticed when the pre-autoclaving pH was lowered from 8.0 to 3.5 (Table 1). After 48 h of growth, regardless of the initial value, the pH in all the growing cultures was near neutrality (Table 1). The bioconversion rates for all the cultures were quasi-linear between 3 and 10 days and increased with decreasing initial pH values (Table 1). The same experiment was repeated with filter sterilized media in order to determine if this effect was due to the formation of a Maillard compound [11]. A similar effect was observed, as initial pH value decreased, bioconversion yield increased (Table 1). Because of the sharp reduction in 27-OH avermectin production observed between the pH values of 3.5 and 3.0, a presterilization pH value of 4.0 was adopted for the subsequent experiments.

Substrate addition

The production of 940 units of 27-OH avermectin versus 735 units was observed when avermectin was added three days versus immediately post inoculation. Addition at three days was adopted on that basis and on the observation that it provided a better reproducibility (data not shown).

Methanol concentrations between 1% and 4% allowed maximum 27-OH avermectin formation while higher concentrations were slightly inhibitory (Table 2). A

TABLE 1

Effect of presterile pH value on the bioconversion of avermectin to 27-OH avermectin[a]

Presterile pH	Autoclave sterilized medium[b]				Filter sterilized medium[c]
	Post-sterile pH	pH at 48 h	27-OH avermectin (units/l)	Bioconversion rate[d] 27-OH avermectin (units/l/day)	27-OH avermectin (units/l)
2.5	2.75	2.80	60	–	0
3.0	3.25	3.30	40	–	0
3.5	3.70	4.00	780	140	0
4.0	4.55	6.80	730	80	598
4.5	5.00	6.90	690	80	507
5.0	5.60	7.00	640	75	484
5.5	5.60	7.00	490	58	336
6.0	6.10	7.10	560	55	431
6.5	6.50	7.15	530	48	492
7.0	6.65	7.20	470	45	333
7.5	6.80	7.10	420	46	227
8.0	7.05	7.05	350	30	212

[a] All data are average of 2 flasks.

[b] The flasks (250 ml containing 20 ml of medium) were autoclaved for 20 min at 121 °C.

[c] The medium was filtrated through an 0.22 μm membrane. The post-filtration pH was unchanged.

[d] The bioconversion rates were calculated by linear regression on data collected between 3 and 10 days. All rates had correlation coefficients > 0.85.

TABLE 2

Effect of methanol concentration on the bioconversion of avermectin to 27-OH avermectin

Methanol final concentration[a] (%)	27-OH avermectin (units/l at 14 days)
1	940
2	1045
4	975
6	740
8	550

[a] The methanol solution containing the avermectin was added 3 days post-inoculation. The final concentration of avermectin was 6250 units/l.

methanol concentration of 4% was adopted for the future experiments as it potentially allows the addition of larger amounts of avermectin.

A linear increase in the production of 27-OH avermectin was observed when the concentration of avermectin increased from 3150 units/l to 12550 units/l (Fig. 3). Nor further increases in the production of 27-OH avermectin were noticed with higher concentrations of substrate. Since higher concentrations did not inhibit the bioconversion process, a concentration of 20000 units/l of avermectin was selected for the next experiments.

Medium composition

A linear increase in the production of 27-OH avermectin was observed when the original concentration of the nitrogen containing medium ingredients (defined as the total concentration of yeast extract plus beef extract, and peptone) was varied while the glucose concentration was kept constant at 10 g/l (Fig. 4). Higher amounts of nitrogen containing substrates were inhibitory to bioconversion. A nitrogen-carbon balance of 1.5 was adopted for medium strength optimization studies (10 g/l glucose, 1.5 g/l beef extract, 3 g/l yeast extract and 4.5 g/l peptone). Higher amounts of 27-OH avermectin were produced when medium strength was increased from 0.75 to 2 fold (Fig. 5). High biomass production was observed in the cultures that produced higher amounts of 27-OH avermectin. Medium strengths above 3.0-fold were almost totally inhibitory to the bioconversion. The final improved medium contained glucose, 20 g/l; yeast extract, 6 g/l; beef extract, 3 g/l and peptone, 9 g/l.

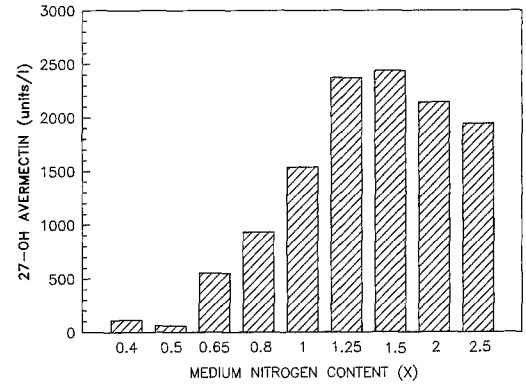

Fig. 4. Effect of medium balance on the bioconversion of avermectin to 27-OH avermectin. X, Total concentration of nitrogen containing ingredients (peptone + yeast extract + beef extract). 1X is the original medium concentration (beef extract, 1 g/l; yeast extract, 2 g/l and peptone, 3 g/l). The carbon source, glucose was kept constant at 10 g/l for all X values.

Scale-up studies

Initial scaled-up fermentations experienced heaving foaming upon substrate addition. When polyglycol-2000 (our standard antifoam) was added, a total inhibition of the bioconversion process was observed. Various antifoams were screened in shake flasks for their degree of

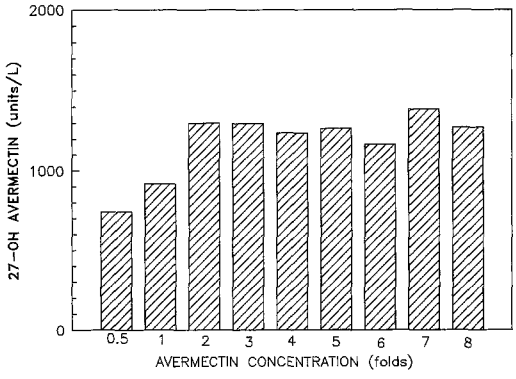

Fig. 3. Effect of avermectin concentration on its bioconversion to 27-OH avermectin. One fold of avermectin represents 6250 units/l.

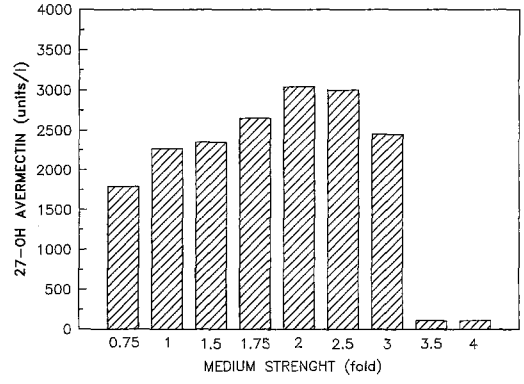

Fig. 5. Effect of medium strength on the bioconversion of avermectin to 27-OH avermectin.

inhibition to the process. Various amounts of 27-OH avermectin were produced (in percent of control) when the following synthetic antifoams were added at the final concentration of 1000 ppm: FD 62 (91%), pluronic L 122 (23%), Tetronic 901 (21%) and Pluracol P2010 (18%). The production of 27-OH avermectin was 61% and 21% of that obtained in controls (no antifoam addition) when lard burning oil and Proflo oil were respectively added at the final concentration of 5 ml/l. The most promising ones (lard burning oil and FD 62) were further evaluated in 14-l fermentors. the concentration of the silicone antifoam FD 62 had to be increased from 1000 ppm to 15000 ppm in order to fully control foaming and resulted in lower bioconversion yields when compared with lard burning oil (330 units/l vs. 410 units/l). This later antifoam was adopted for the large scale fermentations.

For safety purposes, methanol, the solvent used to disperse avermectin had to be replaced with a non-explosive solvent. Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) were screened over the concentration range of 0.4% of 2.0%. DMSO out performed DMF by more than 20% in that concentration range and was selected for the fermentor studies. The titers of 27-OH avermectin obtained with DMSO were almost linear in that range of concentration but were 20% lower than those obtained with methanol when added at the final concentration of 4%.

The scaled-up bioconversions were performed in 800-l fermentors. Because of high air velocity encountered in these fermentors, foaming was still a problem and had to be controlled by reducing the medium strength back to its original value and by increasing lard burning oil concentration to 11 ml/l (5 ml in the medium charge and 6 ml added for foam control during the fermentation cycle). Titers of 250 units/l of 27-OH avermectin were achieved during these fermentations (Fig. 6).

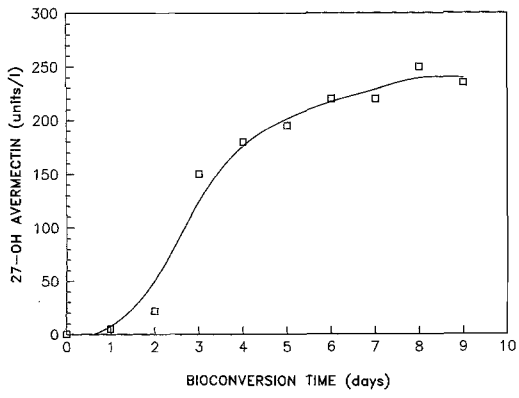

Fig. 6. Time course of avermectin bioconversion to 27-OH avermectin in 800-l fermentor.

DISCUSSION

The above studies resulted in an increase in the volumetric production of 27-OH avermectin in shake flask from 400 units/l to 3200 units/l representing an 8-fold improvement. Parts of this titer improvement can be attributed to an increase in catalyst concentration obtained through optimizing medium balance and strength. Modifications of the fermentation physicochemical environment that stimulated the bioconversion machinery and substrate addition strategy also contributed in titer improvement. After the process was modified to meet stirred vessel requirements, the successfully scaled-up bioconversion produced 27-OH avermectin in quantity suitable for large scale isolation.

The initial pH value of the presterile medium influenced greatly the yield of the bioconversion. This effect was observed with medium sterilized either by autoclaving or by filtration. This seems to rule out any direct effect of heat treatment such as the formation of an stimulatory Maillard compound or the destruction of an acid thermounstable inhibitor [11]. As the pH of the medium decreased, precipitation of previously soluble matter was noticed in media sterilized by both processes. Bioconversions have been reported to be affected by the concentration of salts and heavy metals [4,6,12]. Rather than directly affecting the activity of periplasmic enzymes involved in avermectin bioconversion [14], the pH effect observed on this present bioconversion could be a detoxification of the fermentation medium through precipitation of toxic(s) components. The fact that in this bioconversion, the pH values were significantly different only during the first 48 h while the bioconversion rates were linear between 3 and 10 ways support this hypothesis.

Substrate addition strategy proved to have a large effect on the bioconversion yield. Amounts of avermectin added, time of addition and final concentration of methanol were the critical factors. When the avermectin was added 3 days post inoculation, bioconversion to 27-OH avermectin was usually better than when added immediately post inoculation. This can be attributed to a direct individual or combined toxicity of methanol and avermectin to the cells. No induction by addition of small amounts of avermectin was required in this process. Concentrations of 27-OH avermectin bioconverted were dependent on the amount of substrate added in the range of 3100 to 12500 units/l.

The screening and selection of a suitable antifoam was a key factor in scaling up the bioconversion. The inhibitory effect observed with most of the antifoams can speculatively be attributed to a preferential partitioning of the avermectin in the non-water miscible antifoam phase. As a consequence, the substrate for bioconversion was

unavailable to the cells. The reduction in 27-OH avermectin observed in medium size fermentors was caused by the reduction in medium strength and by an increased concentration in lard burning oil. Both of these modifications were made in order to control heavy foaming encountered in these vessels. A future strategy for increased titers could involve a two cycle bioconversion; first an optimized biomass production phase, and secondly a bioconversion phase using high concentrations of harvested and washed cells in a buffer containing limited amounts of antifoam.

REFERENCES

1 Barragry T.B. 1987. A review of the pharmacology and clinical uses of ivermectin. Can. Vet. J. 28(8): 512–517.

2 Burg, R.W., B. Miller, E. Baker, J. Birnbaum, S. Currie, R. Hartman, Y. Kong, R. Monaghan, G. Olson, I. Putter, J. Tunac, H. Wallick, E. Stapley, R. Oiwa and S. Omura. 1979. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob. Agents Chemother. 15: 361–367.

3 Chabala, J., H. Mrozik, R. Tolman, P. Eskola, A. Lusi, L. Peterson, W. Campbell, J. Ergerton and D. Ostlind. 1980. Ivermectin, a new broad-spectrum antiparasitic agent. J. Med. Chem. 23: 1134–1136.

4 El Refai, A.B., L. Salam and I. EL. Kady. 1970). Transformation of progesterone by *Rhizopus nigricans* REF 129 as influenced by modification of the fermentation medium. Bull. Chem. Soc. Jpn. 43: 2878–2884.

5 Goodhue, C.T. 1982. The methodology of microbial transformations of organic compounds. In: Microbial Transformations of Bioactive Compounds, Vol. 1, (Rosazza, J.P., ed.), pp. 9–44, CRC Press Inc., Boca Raton, FL.

6 Goodman, J.J. and L.L. Smith. 1960. 16 α-Hydroxysteroids. IX. Effect of medium composition on isomerization of 9 α fluoro-16α hydroxyhydro-cortisone and 9α-fluoro-16α hydroxy prednisolone (triamcinolone) during microbiological fermentations. Appl. Microbiol. 8: 363–366.

7 Kieslich, K. 1986. Production of drugs by microbial biosynthesis and biotransformation. Arzneim-Forsch/Drug Res. 36: 888–892.

8 Kieslich, K. 1986. Production of drugs by microbial biosynthesis and biotransformation. Arzneim-Forsch/Drug Res. 36: 774–778.

9 Krasnobajew, V. 1984. Terpenoids. In: Biotechnology, Vol. 6a (Kieslich K., ed.), pp. 97–126, Verlag Chemie, Weinheim, Germany.

10 Leuenberger, H.G.W. 1984. Methology. In: Biotechnology, Vol. 6a (Kieslich, K., ed.), pp. 5–32, Verlag Chemie, Weinheim, Germany.

11 Mauron, J. 1981. The maillard reaction in food; a critical review from the nutritional standpoint. Prog. Food Nutr. Sci. 5: 5–35.

12 O'Connell, P.W., K.M. Mann, E.D. Nielson and F.R. Hanson. 1955. Studies of the microbiological oxidation of steroids by Cunninghamella Blakesleeana M. 334 II. medium design. Appl. Microbiol. 3: 16–20.

13 Okazaki, T., N. Serizawa, R. Enokita, A. Torikata and A. Terahara. 1983. Taxonomy of actinomycetes capable of hydroxylation of ML-236B (compactin). J. Antibiot. 36: 1176–1183.

14 Plourde, R. and H. Hafez Zedan. 1973. Distribution of steroid 1-dehydrogenation and side chain degradation enzymes in the spores of *Eusorium solani*: Causes of metabolic lag and carbohydrate independence. Appl. Microbiol. 25: 650–658.

15 Robson, I.G. and P. Baker. 1988. The potential for biotransformations in specialty chemical manufacture. Spe. Chem. 8: 239–254.

16 Springer, J., B. Arison, J. Hirshfield and K. Hoogsteen. 1981. The absolute stereochemistry and conformation of avermectin B2a Aglycon and Avermectin Bla. J. Am. Chem. Soc. 103: 4221–4224.

17 Vingtain, P., E. Pichard, J. Ginoux, S.M. Coulibaly, Y. Bissan, P. Ranque and B. Thillaye. 1988. Ivermectin and human onchocerciasis: a study of 234 patients in Mali. Bulletin de la Societe de Pathologie Exotique, et de ses filiales 81: 260–270.