Biotransformation of Selamectin with *Streptomyces lydicus* SX-1298 Using a Novel Static Agar Fermentation System with Reemay® Mesh

MICHAEL S. PACEY[*,†], MARION M. BARNES[†], ROBERT A. MONDAY[†], MICHAEL RITZAU[††] and ELIZABETH M. H. WELLINGTON[†††]

[†] Veterinary Medicine Discovery, Pfizer Global Research and Development, Sandwich, Kent, UK
[††] Department of Structural and Separation Sciences, Pfizer Global Research and Development, Sandwich, Kent, UK
[†††] Department of Biological Sciences, University of Warwick, Coventry, UK

(Received for publication January 22, 2001)

A novel approach to biotransformation is described using a solid medium matrix and Reemay® mesh that gives efficient biotransformation of compounds with minimal matrices in the ensuing gum solids. Using this approach with a newly isolated biotransforming organism, *Streptomyces lydicus* SX1298, a series of hydroxylations and an *O*-demethylation is described for selamectin the first endectocide for cats and dogs.

Selamectin, (5*Z*,13*S*)-α-oleandrosyl-25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5-(hydroxyimino)-avermectin B1 monosaccharide is active against fleas and ticks, intestinal hookworms, roundworms and immature heartworm[1] and has been commercialised as Revolution® (U.S.A.) and Stronghold® (Europe), an endectocide for companion animals. In order to explore the structure activity relationship around selamectin to give compounds not readily accessible by semi-synthesis, a biotransformation programme in submerged culture was embarked upon with very little success. Single figure milligram amounts of biotransformed compounds were present in grams of gum solid. A novel method is reported where a solidified biotransformation medium is used and the compound is impregnated on to a sterile polyester Reemay® mesh through which the organism grows. This results in efficient biotransformations with minimal media matrix in the ensuing gum solid. Here we describe the resulting biotransformation products of selamectin using this novel method with a newly isolated biotransforming organism, characterised as *Streptomyces lydicus* SX1298.

Materials and Methods

Taxonomy

The organism, SX-1298 was isolated from a soil sample collected in Sandwich, Kent, U.K. It was maintained on agar containing one quarter strength ATCC172 medium. The culture has a characteristic grey spore mass that readily lyses with age but appears black as spore chains are covered in a mucous coating. No mycolic acids were found to be present[2] and LL-diaminopimelic acid was detected in the whole-cell hydrolysate of the strain. The fatty acid profile of the cell wall shows that the major components present are branched *iso* and *anteiso* acids together with some straight chain acids. These are summarised as follows:

Acid type	Acid	Ratio in cell wall
Iso	13-Methyltetradecanoic acid	0.25
Anteiso	12-Methyltetradecanoic acid	1.00
Iso	14-Methylpentadecanoic acid	0.77
Straight chain	Hexadecanoic acid	0.25
Iso	15-Methylhexadecanoic acid	0.18
Anteiso	14-Methylhexadecanoic acid	0.57

* Corresponding author: mike_pacey@sandwich.pfizer.com

Characterisation of the *Streptomyces* species was performed using the forty one morphological and physiological diagnostic characters contained in the probabilistic identification matrix of WILLIAMS *et al.*[3] Three identification statistics were used to assess the reliability of the identification of unknown strains. The Willcox probablity was used to assign an identity to an unknown, where scores of 0.8 and above indicated a positive identification. Taxonomic distance (d) determined the distance of the unknown from the centroid of the cluster group to which the unknown had been assigned; scores of 0.4 and below were indicative of a position within the cluster. The standard error of d gave a measure of the variation in test results of the unknown around the centroid of a cluster with which it was identified; scores of 2.0 or less were typical of strains grouped within the cluster and a negative score indicated the unknown was closer to the centroid than average.

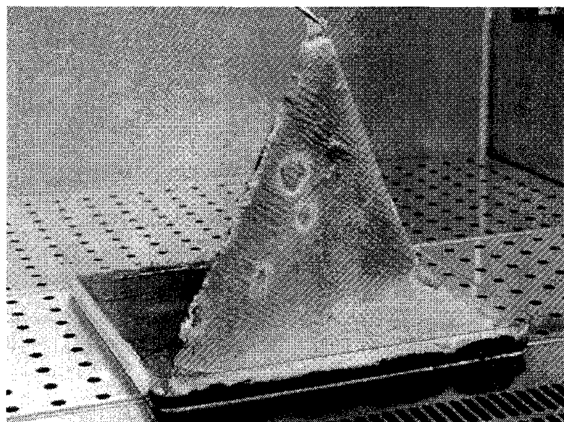
The strain identified with the *Streptomyces lydicus* species group as described in BERGEY's manual.[4] The identification score for the Wilcox probability was 1.0 and the taxonomic distance 0.418 indicating some atypical characters which included inability to utilise adonitol, and resistance to azide and neomycin. It has been deposited at the American Type Culture Collection and assigned the Accession Number PTA-1687

Fermentation

A slant culture of *S. lydicus* SX-1298 maintained on one quarter strength ATCC172 agar was used to inoculate a 300 ml Erlenmeyer flask containing 50 ml of a seed medium containing cornstarch 2%, cotton seed meal 1.5% (Pharmamedia®), nitrogen source Ardamine pH® 0.5% and $CaCO_3$ 0.2% in tap water at a natural pH of 7.2. The flask was incubated for 72 hours at 28°C on a rotary shaker with one inch throw.

Ten biotransformation production plates were prepared as follows. Cornstarch 8%, yeast extract (Oxoid®) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, glutamic acid 0.06%, $FeSO_4 \cdot 7H_2O$ 0.01%, $ZnSO_4 \cdot 2H_2O$ 0.0001%, $MnSO_4 \cdot H_2O$ 0.0001%, was dissolved in 3 litres of tap water and agar 2% added. It was sterilised at 121°C for 30 minutes in six 500 ml Duran bottles and then allowed to cool to 60°C before pouring the molten agar, 250 ml into each of ten sterile 24×24 cm Nunc® biossay dishes (Part No 240835). Selamectin, 100 mg was dissolved in methanol, 20 ml, and used to impregnate ten 23×23 cm Reemay 2250® mesh (Nordlys SA, 59270 Bailleul, France) by dipping each sterile mesh in the solution and allowing to dry in the airstream of the Hereaus® microbioligical cabinet. Each impregnated mesh was then laid on to the solid medium matrix on the plate and then inoculated with 20 ml of the seed described above. The culture was then evenly spread across each plate and then allowed to incubate at 28°C for 14 days until the culture had fully sporulated. The plate was then cooled to 4°C before each mesh was removed for extraction leaving the solid medium matrix behind. (Fig. 1)

Fig. 1. Reemay mesh at harvest containing the biotransformation products leaving the agar medium matrix behind.



Isolation

The ten meshes were removed and extracted with methanol (2×2l), evaporation of which gave a crude mixture of biotransformation products (7.1 g). This was suspended in heptane (150 ml) and the heptane solubles decanted off and discarded. The heptane insolubles were then taken to dryness (306 mg). This was dissolved in acetonitrile (5 ml) and then one millilitre aliqouts injected on to a Phenomonex Magellan® reversed-phase semi prep HPLC column (150 mm×21.7 mm, Part No. 00F-4257-P0). It was then eluted using a gradient of acetonitrile-water 70 : 30 from 0 to 2 minutes, 70 : 30 to 95 : 5 from 2 to 8 minutes and 95 : 5 from 8 minutes to 18 minutes returning to the original conditions from 18 minutes to 20 minutes. The flow rate was 20 ml/minute and detection was by UV at 243 nm. From the five runs, samples with the same retention time and mass ion were combined to give two single peak components (*ca.* 100 μg each, peak **3** and peak **5** in Table 1) which were then characterised.

Characterisation and Structure Elucidation

The crude mixture of biotransformation products was characterised by LC-MS and LC-NMR-MS. LC-MS

analysis was performed using a Phenomonex Luna® reversed-phase analytical column (50×2 mm), flow rate 1 ml/minute, using a linear gradient from 10:90 to 98:2 acetonitrile-ammonium formate (0.05%) buffer over 2.67 minutes, and then maintained at 98:2 for a further 1.33 minutes (System A). The LC-MS data was collected on an Finnigan AQA instrument.

For the LC-NMR-MS analysis a linear gradient over 40 minutes from 18:82 to 97:3 CD_3OD-ammonium formate-d_7 (0.05% in D_2O) buffer was used (System B). The LC-NMR spectra were acquired in stopped flow mode on a Varian INOVA spectrometer at 600 MHz proton frequency using a 60 μl inverse detection flow probehead and the MS spectra on a Finnigan Navigator single quadrupole mass spectrometer in ES positive ion mode. The NMR spectra of the isolated peaks **3** and **5** were acquired in DMSO-d_6 using an inverse detection 1.7 mm capillary probe. Chemical shifts (δ) are in ppm relative to CD_3OD and DMSO-d_6, respectively.

Results and Discussion

Table 1 summarises the LC-MS and LC-NMR-MS data for the crude biotransformation mixture.

The available sample amount allowed the acquisition of LC-WET ^{1}H and LC-WET TOCSY spectra for all peaks.[5] The online data clearly reveal a pattern of demethylation of the 3′-OMe and hydroxylation of the cyclohexyl ring. Whilst the 3′ demethylation was easily and unambiguously proven for **1**, **3** and **5** (lack of 3′-OMe signal and downfield shift of 3′-H), the position of the hydroxylations in **1**~**4** could only be narrowed down to the cyclohexyl moiety. Severe signal overlap and the limited sample amount prevented the exact determination of the hydroxylation sites under LC-NMR conditions.

Hence the main peaks **3** and **5** were isolated and their structure elucidated by mass spectrometry (Fig. 3 for **3**) and NMR spectroscopy. Proton, DQF-COSY, TOCSY, HSQC

Table 1. LC-MS/LC-NMR-MS characterisation of the biotransformation products.

Peak	Rt (A)	Rt (B)	Mass M+H$^+$	Mass M+D$^+$	Number of exchangeable protons	Interpretation
1	3.17	8.44	788	795	6	Cyclohexane ring dihydroxylated, 3′-OMe→OH
2	3.40	14.82	802	808	5	Cyclohexane ring dihydroxylated
3	–	15.42	772	778	5	Cyclohexane ring monohydroxylated, 3′-OMe→OH
4	–	19.17	786	791	4	Cyclohexane ring monohydroxylated
5	3.54	21.92	756	761	4	3′-OMe→OH
6	3.70	26.64	770	774	3	Selamectin

Fig. 2. Structures of selamectin, and its biotransformation products **3** and **5**.

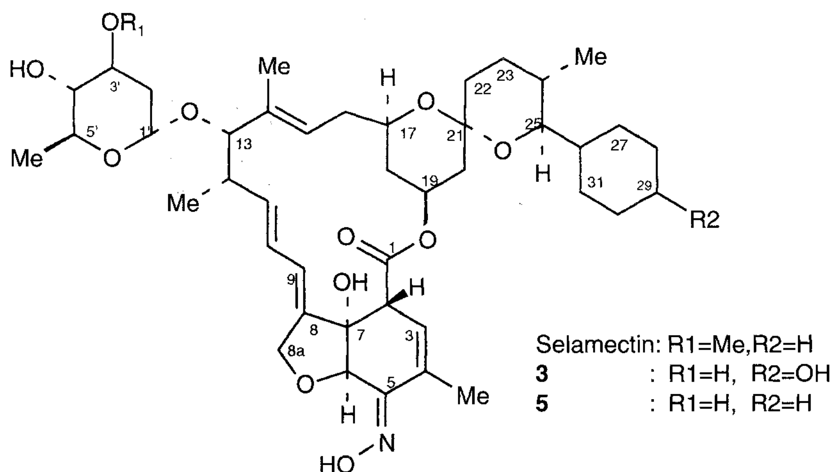


Table 2. ^{1}H NMR chemical shifts for selamectin, **3** and **5** in DMSO-d_6.

Proton	Selamectin	**3**	**5**
2	3.18 (m)	3.17 (m)	3.17 (m)
3	5.80 (m)	5.80 (m)	5.80 (m)
6	4.39 (s)	4.38 (s)	4.38 (s)
8a	4.52 (s, br)	4.52 (s, br)	4.51 (s, br)
9	5.79 (dt, 11.4, 2.4)	5.78 (dt, 11.4, 2.4)	5.79 (dt, 11.4, ~2)
10	5.88 (dd, 14.8, 11.4)	5.87 (dd, 14.8, 11.4)	5.87 (dd, 14.5, 11.4)
11	5.61 (dd, 15.0, 9.8)	5.58 (dd, 15.0, 10.0)	5.58 (dd, 15.0, 10.0)
12	2.62 (m)	2.62 (m)	2.61 (m)
13	3.89 (s, br)	3.89 (s, br)	3.89 (s, br)
15	5.09 (d, br, ~11)	5.07 (d, br, ~11)	5.05 (d, br, ~11)
16	2.17 (m)	2.18 (m)	2.18 (m)
	2.23 (d, br, ~12)	2.23 (d, br, ~12)	2.23 (d, br, ~12)
17	3.59 (ddt, 11, 4.7, 2)	3.59 (m)	3.60 (m)
18	0.74 (ax) (t, br, 12.1)	0.73 (ax) (t, br, 12.1)	0.73 (ax) (t, br, 12.1)
	1.80 (eq) (d, br, 12.1)	1.79 (eq) (m)	1.78 (eq) (d, br, ~12)
19	4.93 (m)	4.92 (m)	4.92 (m)
20	1.15 (ax) (m)	1.14 (ax) (m)	1.14 (ax) (m)
	2.05 (eq) (ddd, 11.8, 5.0, 1.5)	2.05 (eq) (ddd, 11.8, 5.0, 1.5)	2.07 (eq) (ddd, 11.8, 5.0, ~1.5)
22	~1.4–1.6 (m)	~1.4–1.6 (m)	~1.4–1.6 (m)
23	1.43 (m)	1.43 (m)	1.44 (m)
24	1.45 (m)	1.45 (m)	1.48 (m)
25	3.05 (d, br, ~9)	3.05 (d, br, ~9)	3.07 (d, br, ~9)
26	1.50 (m)	1.50 (m)	1.50 (m)
27	1.63–1.78 (m)	1.63–1.78 (m)	1.89 (m)*
	1.10–1.24 (m)	1.10–1.24 (m)	1.12 (m)*
28	1.63–1.78 (m)	1.63–1.78 (m)	1.74 (d, br, ~13)**
	1.10–1.24 (m)	1.10–1.24 (m)	1.41 (m)**
29	1.63–1.78 (m)	1.63–1.78 (m)	3.84 (m, br)
	1.10–1.24 (m)	1.10–1.24 (m)	
30	1.59 (m)	1.59 (m)	1.69 (d, br, ~13)**
	1.15 (m)	1.15 (m)	1.33 (m)**
31	1.63–1.78 (m)	1.63–1.78 (m)	1.58 (m)*
	1.10–1.24 (m)	1.10–1.24 (m)	1.28 (m)*
4-Me	1.84 (s, br)	1.84 (s, br)	1.83 (s, br)
12-Me	1.08 (d, 6.9)	1.08 (d, 6.9)	1.08 (d, 6.9)
14-Me	1.46 (s, br)	1.46 (s, br)	1.47 (s, br)
24-Me	0.75 (d, br, 5.3)	0.75 (d, br, 5.3)	0.75 (d, br, 5.3)
1′	4.69 (m, br)	4.65 (m, br)	4.65 (m, br)
2′	1.42(ax) (m)	1.51(ax) (m)	1.51(ax) (m)
	2.16 (eq) (d, br, ~13)	1.94(eq) (dd, 13.7, 4.9)	1.93(eq) (dd, 13.7, 4.9)
3′	3.36 (m)	3.64 (m)	3.64 (m)
4′	2.89 (dt, 9.0, 5.5)	2.78 (dt, 9.0, 5.4)	2.78 (dt, 9.0, 5.4)
5′	3.67 (dq, 9.4, 6.2)	3.65 (m)	3.65 (m)
3′-OMe/OH	3.33 (s)	4.73(OH) (d, 4.8)	4.73(OH) (d, 4.8)
5′-Me	1.12 (d, 6.2)	1.12 (d, 6.2)	1.12 (d, 6.2)
5-NOH	11.48 (s)	11.48 (s)	11.48 (s)
7-OH	5.28 (s)	5.29 (s)	5.29 (s)
29-OH	–	–	4.14 (br)
4′-OH	5.05 (d, 5.5)	4.93 (d, 5.4)	4.94 (d, 5.4)

*/** The assignment of 27-CH_2/31-CH_2 and of 28-CH_2/30-CH_2 is interchangeable.

Fig. 3. Positive ion mode ES-MS of **3** under deuterium exchange conditions.

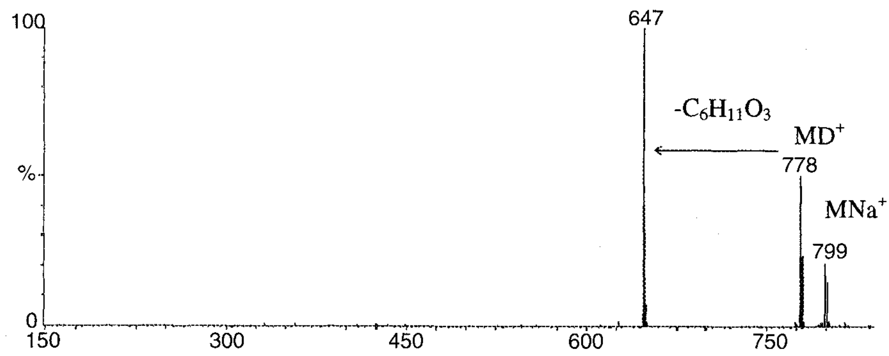


Fig. 4. ^{1}H-NMR spectrum in DMSO-d_6 of a) Selamectin and b) **3**.

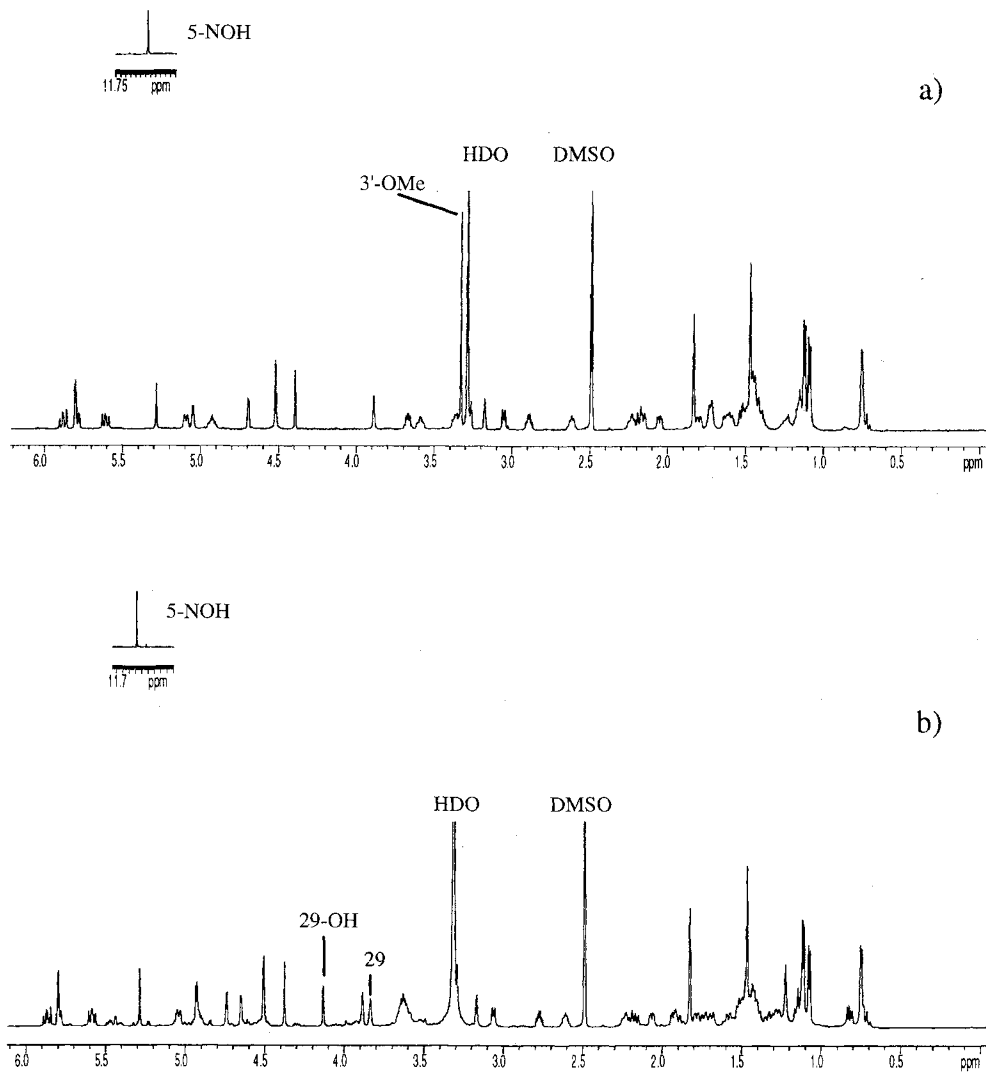
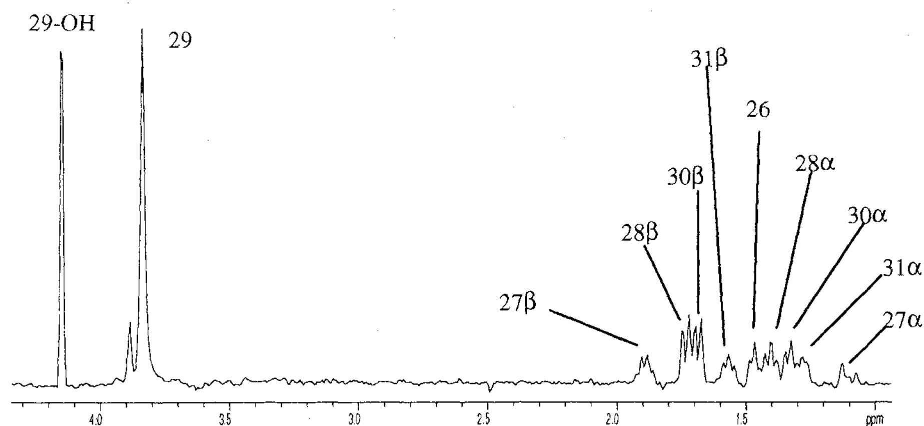


Fig. 5. Row at δ_H 3.84 of the TOCSY spectrum of **3** in DMSO-d_6.



and HMBC data were acquired for both. The NMR assignments are given in Table 2. In the ^{1}H NMR spectrum of **3** (Fig. 4b) and **5** the signal of 3′-OMe is replaced by a new exchangeable doublet at δ_H 4.73 coupled to a signal at δ_H 3.64 that was identified as 3′-H. This provides clear evidence that 3′-OMe has been transformed into a hydroxyl group in **3** and **5**. Both NMR chemical shifts and mass spectroscopic data showed that **5** is otherwise unchanged. In the case of **3** two additional downfield signals which are coupled to each other were observed at δ_H 4.14 and δ_H 3.84 (Fig. 4). The former signal exchanges with D_2O. These data, in addition to the MS results suggested the hydroxylation of a CH_2 group in **3**. The position of this hydroxylation was determined as C-29 after analysis of the DQF-COSY and TOCSY (row at δ_H 3.84 (29-H) see Fig. 5) spectra. The fact that the methylene signal for C-27/31 and C-28/30 are not equivalent suggests a hindered rotation of the 1,4-substituted cyclohexanol moiety due to the presence of C-24 methyl group.

Submerged fermentation processes for biotransformation are often inefficient due to catabolite repression at the carbon source for enzyme production and lack of specificity of the biotransforming enzyme for the substrate. Moreover, the resulting gum solids containing the biotransformation products are rich in unwanted media components. Pectinase production by solid state fermentation studies have shown that extracellular enzyme production by *Aspergillus niger* was not affected by catabolite repression at carbon source or inducer concentrations at 100 g/liter.[6] Pectinase activity and productivity were up to 10 and 20 times higher respectively than those obtained in the submerged fermentation system. It is this increase in enzyme activity that is believed responsible for the much greater biotransformation efficiency of this novel static agar system when compared with the submerged process. Moreover because the solid medium matrix is left behind there are relatively few medium components in the ensuing biotransformation product rich gum solid.

Both **3** and **5** showed at least 10 fold reduced activity in a flea *in vitro* assay[7] compared with selamectin.

References

1) BISHOP, B. F.; C. I. BRUCE, N. A. EVANS, A. C. GOUDIE, K. A. F. GRATION, S. P. GIBSON, M. S. PACEY, D. A. PERRY, N. D. A. WALSHE & M. J. WITTY: Selamectin: A novel broad spectrum pet endectocide. Vet. Parasitol. 91: 163~176, 2000
2) BAXTER, A. J.: Identification of culture SX-1298, The National Collections of Industrial and Marine Bacteria Limited, Aberdeen, U.K.
3) WILLIAMS, S. T.; M. GOODFELLOW, E. M. H. WELLINGTON, J. C. VICKERS, G. ALDERSON, P. H. A. SNEATH, M. J. SAKIN & A. M. MORTIMER: A probability matrix for identification of some streptomycetes. J. Gen. Microbiol. 129: 1815~1830, 1983b
4) WILLIAMS, S. T.; M. E. SHARPE & J. G. HOLT: BERGEY'S Manual of Sytematic Bacteriology, Vol.4, Williams and Wilkins, Baltimore, 1989
5) SMALLCOMBE, S. H.; S. L. PATT & P. KEIFER: WET solvent suppression and its applications to LC-NMR and high-resolution NMR spectroscopy. J. Magn. Reson. A. 117: 295~303, 1995
6) HUERTA, S.; E. FAVELA, R. LOPEZ-ULIBARRI, G. VINIEGRA-GONZALEZ & M. GUTIERREZ-ROJAS: Absorbed substrate fermentation for pectinase production with *Aspergillus niger*. Biotechnol. Tech. 8(11): 837~842,

1994

7) BANKS, B. J.; B. F. BISHOP, N. A. EVANS, S. P. GIBSON, A. C. GOUDIE, K. A. F. GRATION, M. S. PACEY, D. A. PERRY & M. J. WITTY: Avermectins and flea control: structure-activity relationships and the selection of selamectin for development as an endectocide for companion animals. Bioorg. Med. Chem. 8(8): 2017~2025, 2000