FERMENTATION AND PURIFICATION METHODS FOR THE PRODUCTION OF LEPTOMYCIN B AND RELATED ANALOGS FROM *STREPTOMYCES SP.*

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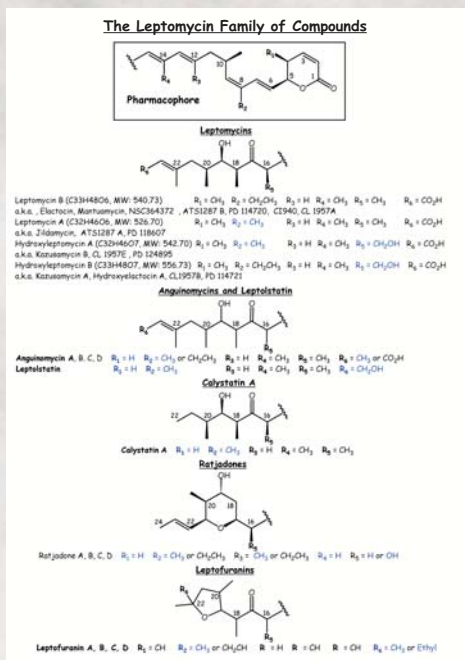
Introduction

In 1983 leptomycin A (LPT A) and leptomycin B (LPT B) were discovered at the University of Tokyo as potent anti-fungal agents against ***Schizosaccharomyces pombe***, ***Mucor racemosus*** and ***Mucor rouxiannus*** (ref 1). In 1985, LPT A, LPT B and the mono-hydroxylated derivatives, kazusamycin A (30-hydroxyleptomycin B, HLPT B) and kasuzamycin B (29-hydroxyleptomycin A, HLPT A) were isolated and reported as antitumor compounds (ref 2).

Since then several compounds that share similar structural features and biological activities have been isolated from other sources (Figure 1), including the anguinomycins (from Streptomyces sp. R2827) (ref 3), leptolstatin (from Streptomyces sp. SAM 1595) (ref 4), callystatin A (from a marine sponge ***Callyspongia truncata***) (ref 5), the ratjadones (from a myxobacteria, ***Sporangium cellulosum***) (ref 6) and the leptofuranins (from Streptomyces sp.) (ref 7).

Figure 1. Related compounds known as the leptomycin family that share similar structural and biological features.



Biological Target of the Leptomycins (Figure 2)

The life cycle of a cell depends on the transport of proteins through the nuclear pore complex (NPC), the gateway that separates the nucleus/cytoplasm contents of the cell. This gateway contains aqueous channels that allow molecules, up to 50kDa, to pass freely in and out of the nuclear envelope. Larger macromolecules, such as RNA or proteins with diameters up to 28nm, must be activated by a shuttling transport factor in order to pass through the gateway (ref 8).

The chromosomal regional maintenance 1 protein (CRM1) is one of several shuttling factors that bind to proteins, which have a leucine-rich nuclear export signal sequence (LR-NES), allowing these proteins to be transported through the nuclear envelope. Studies show that LPT B interacts with CRM1 and interferes with the function of this shuttling protein (ref 9). Other members of the Leptomycin family behave similarly, allowing the identification of the pharmacophore responsible for this interaction, as the α, β-unsaturated cyclic lactone with the unsaturated polyketide chain containing the two diene moieties (Figure 1). The various members of this family will allow researchers to identify and understand CRM1-mediated processes and determine whether CRM1 is a new target for antitumor chemotherapy.

LPT B, LPT A and HLPT B have demonstrated promising in vitro cytotoxic activity against several human and mouse tumor cell lines at very low dosages compared to three clinically used antitumor agents, Adriamycin, Cisplatin and Methotrexate. Unfortunately, the mouse xenograft models showed adverse side effects such as malaise and anorexia (ref 10, table1). To pursue the potential of the leptomycins as new anticancer drugs, a source for these compounds was required. Only leptomycin B is commercially available, from Sigma ($340 per 0.5μg) and Calbiochem ($295 per 1.0μg). Therefore, the Natural Products Support Group, SAIC-Frederick, Inc., was asked by the Developmental Therapeutics Program, NIH, to investigate the possibility of producing milligram quantities of LPT A, LPT B, HLPT A and HLPT B.

Figure 2. CRM1 interacts with the leucine rich nuclear export sequence on the cargo protein in preparation for exportation through the nuclear pore complex into the cytoplasm.

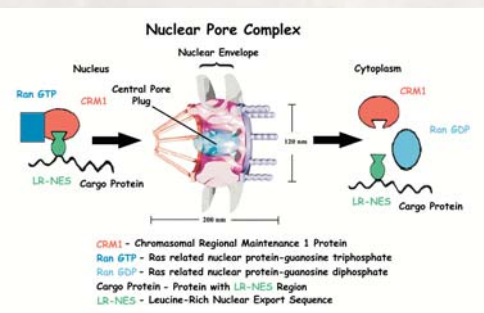


Table 1. In vitro cytotoxicity data of LPT B, HLPT B and LPT A compared to three clinically used antitumor agents (ref 10).

Tumor Cell Line	IC 50 (nM)					
	Leptomycin B	Hydroxy-leptomycin B	Leptomycin A	Adriamycin	Cisplatin	Methotrexate
L1210 Leukemia	0.22	0.33	0.17	41	1100	21
Lewis Lung Carcinoma	0.37	1.6	0.58	250	640	66
Pancreatic Carcinoma 02	2.8	8	3.4	71	1500	89
Mammary Adenocarcinoma 16/C	0.61	-	0.97	38	220	7.3
HCT-8 Human Colon	0.37	1.7	0.51	290	1300	14
A549 Human Colon	0.45	1.2	0.54	90	22000	3600
MCF-7 Human Mammary Carcinoma	0.19	0.66	0.27	91	3900	180

IC 50 - Concentration of drug that will reduce the tumor cell growth to 50% of the control value

Instruments and Procedures

HPLC/MS Analytical System

Waters/Micromass multidetector analytical system consisting of a Waters Delta 600 gradient pumping system, Waters 2996 photodiode array detector (monitoring at λ = 250nm), Waters Micromass ZQ electrospray ionization mass spectrometer and a Sedere Sedex 75 Evaporative Light Scattering Detector. Data processing was done using the Millennium 32 chromatography manager and Masslynx (version 3.5) for analyzing mass spectral data. The analytical HPLC column was purchased from Varian Analytical, Dynamax 60A C18 silica gel HPLC column (column dimensions: 4.6 x 250mm, pore size: 60A, particle size: 8μm).

Preparative HPLC and MPLC Chromatographic System

Waters HPLC preparative chromatographic system consisting of Waters Delta Prep gradient pumping system, Waters 996 photodiode array detector, Waters 717 Sample manager and an ISCO Foxy 200 fraction collector. The preparative HPLC column was purchased from Varian Analytical, Dynamax 60 C18 silica gel HPLC column (column dimensions: 21 x 250mm, pore size: 60A, particle size: 8μm).

For MPLC a pressure relief valve was installed between the HPLC pump and the glass column to release the pressure at 200psi. The silica gel was preparative grade purchased from EM Science (pore size of 60A, particle size ranging between 60-120μm).

Chromatographic Methods (Figure 3)

HPLC Analysis: A standard of LPT B (NSC 364372-P/5) was obtained from the National Cancer Institute's pure compound library and analyzed for purity and identity by HPLC/MS. The cell extracts and standards were dissolved in methanol to give the appropriate concentration for analysis, 10μL were injected into the column. The flow rate was set to 1.0mL/min and the eluent was monitored at 250nm. Four solvent systems were used to analyze fermentation broths and column fractions; Solvent system A [acetonitrile-0.1M ammonium acetate (65:35), pH adjusted to 5 with acetic acid], gave retention times of 6.2, 7.8, 14.8 and 20.2 minutes for HLPT A, HLPT B, LPT A and LPT B, respectively. Solvent system B [acetonitrile-0.1M ammonium acetate (50:50), pH adjusted to 5 with acetic acid], gave retention times of 14.8 and 21.0 minutes for HLPT A and HLPTB, respectively. Solvent system C [methanol-water trifluoroacetic acid (75:25:0.1)], gave retention times of 11.8 and 15.6 minutes for HLPT A and HLPTB, respectively. Solvent system D [methanol-water trifluoroacetic acid (80:20:0.1)], gave retention times of 14.5 and 18.2 minutes for LPT A and LPTB, respectively.

All four leptomycins have a strong UV maximum at 233nm with a small shoulder at 296nm. The detection limit for leptomycin B was in the range of 0.5 to 1.0mg/L when monitoring at 250nm. A calibration curve was constructed and found to be linear between 0.1 and 100.0mg/L. This was used to estimate the titer of LPT B in fermentations.

TLC Analysis: Fractions were dissolved in dichloromethane and spotted onto an EM Merck Science silica gel F260 TLC plate (10 x 10 cm) and developed in ethyl acetate-dichloromethane (50:50). Leptomycins could be detected by short wavelength (254nm) UV or by spraying with sulfuric acid in ethanol (10:90) and heating the plate. The Rf values for the hydroxyleptomycins and leptomycins were 0.22 and 0.44, respectively.

MPLC Chromatography: The crude organic extract was adsorbed onto silica gel (one part sample to two parts silica gel) using DCM and the solvent was removed by rotary evaporation to give a fine dry powder. The sample was dry-packed into the Michel-Miller glass column and the remaining void was filled with fresh silica gel (about 1 part sample layer to 25 parts fresh silica gel).

Preparative HPLC: Samples were dissolved in methanol to give a concentration of 100mg/mL with 200μL injected into the column. The flow rate was set to 10mL/min and the eluent was monitored at 250nm. Two solvent systems were used to purify the hydroxyleptomycins and leptomycins; Solvent system E [acetonitrile-water-trifluoroacetic acid (50:50:0.1)] gave retention times of 37.3 and 43.3 minutes for HLPT A and HLPT B. Solvent system F [acetonitrile-water-trifluoroacetic acid (65:35:0.1)] gave retention times of 25.3 and 33.3 minutes for LPT A and LPT B

Figure 3a. The enriched organic extract from Streptomyces sp. ATCC 39366 contained all four of the leptomycin compounds. The flow rate was maintained at 1.0 mL/min and the eluent (solvent system A) was monitored at 250nm.

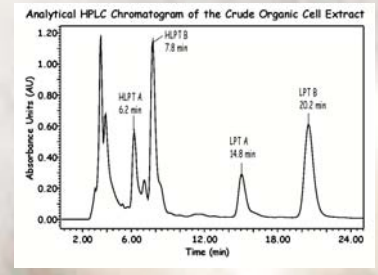
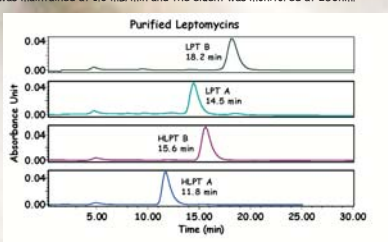


Figure 3b. The purity of the isolated leptomycin compounds was determined by analytical HPLC; Solvent system C was used to analyze HLPT A (93% purity) and HLPT B (94% purity). System D was used to analyze LPT A (85% purity) and LPT B (92% purity). The flow rate was maintained at 1.0 mL/min and the eluent was monitored at 250nm.



The Search for a Producing Organism (Table 2)

A review of the literature led to several leptomycin-producing strains: Streptomyces sp. ATS 1287 (University of Tokyo), Streptomyces sp. No. 81-484 (The Kitasato Institute), ***Streptomyces aburaviensi*** ATCC 39290 (Bristol-Myers), ***Streptomyces pulveraceus***, subsp. ***fostreus*** ATCC 31906 and Streptomyces sp. ATCC 39366 (both from Warner-Lambert/Parke-Davis). We purchased Streptomyces sp. ATCC 39366 and ***Streptomyces aburaviensi*** ATCC 39290 from the American Type Culture Collection (ATCC), fermented both in small volume shake flasks with Bristol-Myers production media recipe (ref 11), then added an equal volume of methanol, homogenized and filtered, then analyzed by HPLC. Streptomyces sp., ATCC 39366 produced LPT B, along with the other three leptomycins (Table 2 and Figure 3). Methods development studies were then initiated with this strain to optimize growth and leptomycin production.

Time and Media Studies (Table 3)

Simple media and time course studies were done. The substitution of an organic nitrogen source from non-hydrolyzed cottonseed embryo meal (Agrilife Industries) with hydrolyzed cottonseed embryo meal (DVM industries), or substituting sodium nitrite for ammonium sulfate as the inorganic nitrogen source both proved detrimental to the production of LPT B.

The concentration of glucose and glycerin was altered. When glucose was removed from the media, an extremely poor yield of LPT B was observed, but removal of glycerin had only a small effect. When both glucose and glycerin were present, increased production of LPT B was found. This led us to believe that glucose was the primary carbon source for this organism and as long as the cells flourished, LBT B titer would increase.

The above studies were carried out on a 10-day fermentation period with daily aliquots removed and analyzed for the presence of LPT B. After a 10-day fermentation period, the titer of LPT B was still increasing. Therefore, future fermentations were grown until all of the glucose was consumed, which was typically about 24 days.

The producing organism was grown successively in larger shake flasks, with production phase consisting of 6 x 6 liter baffled Erlenmeyer flasks, shaking at 230 rpm, fermenting at room temperature for 24 days. The organism grows into a thick, gray paste, which has a tendency to adhere near the top of the flask. Occasional vigorous agitation to remove the accumulation of stuck cells had a positive effect on the cell density and in effect the final yield of the leptomycins.

Brystol-Myers Production Media (ref 12)

20g Glucose
20g Glycerol
10g Soy Flour
10g Non-hydrolyzed Cotton Seed Embryo Meal (Agrilife Industries)
1.0g Ammonium Sulfate
10g Calcium carbonate

Table 2. Streptomyces sp. ATCC 39366 and Streptomyces sp. ATCC 39290 were fermented in Bristol-Myers production media (ref 12). Two things to note; Streptomyces sp. ATCC 39290 is a poor LPT producer and the substitution of the organic nitrogen source with hydrolyzed cottonseed meal from DVM industries significantly lowered the LPT titer.

Organism	Brystol-Myers Media	Harvest Volume (mL)	Leptomycin B HPLC titre (mg/L) Day 7	9	11	12
Streptomyceles sp. ATCC 39366	with Agrilife non-hydrolyzed cotton seed embyo meal	250	24.9	30.7	32.8	23.1
Streptomyceles sp. ATCC 39366	with DVM hydrolyzed cotton seed embyo meal	250	2.5	3	0.7	3.3
Streptomyceles aburaviensi ATCC 39290	with Agrilife non-hydrolyzed cotton seed embyo meal	250	0	0	0	0
Streptomyceles aburaviensi ATCC 39290	with DVM hydrolyzed cotton seed embyo meal	250	0	0	0	1.5

Growth Conditions: 250 mL of production media was placed in a 1 L baffled erlenmeyer shake flask and inoculated with 20 mL of inoculum. The cultures were kept at 28°C (RT), under atmospheric pressure with an agitation rate of 250 rev/min.

Table 3. Streptomyces sp. ATCC 39366 was fermented in Bristol-Meyer's production media with non-hydrolyzed cottonseed meal from Agrilife Industries. No significant changes were noticed in the inoculum amounts but substitutions in the quantity of the carbon sources, glucose and glycerin, showed noticeable changes in the LPT titers. Clearly the best choice for an inorganic nitrogen source is ammonium sulfate and not sodium nitrate.

Organism	Bristol-Myers Media Change	Harvest Volume (mL)	Leptomycin B HPLC Titre (mg/L) Days 4	5	6	7	8	9	10
Streptomyceles sp. ATCC 39366	10% (v/v) inoculum	250	1.3	5.6	6.3	14.6	15.9	28.3	32.2
Streptomyceles sp. ATCC 39366	4% (v/v) inoculum	250	0.4	4.8	6.6	11.1	26.5	36.8	38.8
Streptomyceles sp. ATCC 39366	Excess glucose (30gr)	250	0.4	3.3	10.8	13.4	24.2	37.6	52.5
Streptomyceles sp. ATCC 39366	Excess glycerin (30gr)	250	0.2	2.4	5.0	10.6	18.9	25.9	39.6
Streptomyceles sp. ATCC 39366	no glycerin	250	0.0	2.5	4.1	5.6	8.8	14.4	27.4
Streptomyceles sp. ATCC 39366	no glucose	250	0.0	0.8	2.4	5.0	7.8	6.9	9.1
Streptomyceles sp. ATCC 39366	with sodium nitrite	250	0.0	0.7	4.5	7.1	7.3	10.7	16.6

Growth Conditions: 250 mL of production media was placed in a 1 L baffled erlenmeyer shake flask and inoculated with 20 mL of inoculum. The cultures were kept at 28°C (RT), at atmospheric pressure with an agitation rate of 250 rev/min.

Extraction and Purification (Figure 3 and Figure 4)

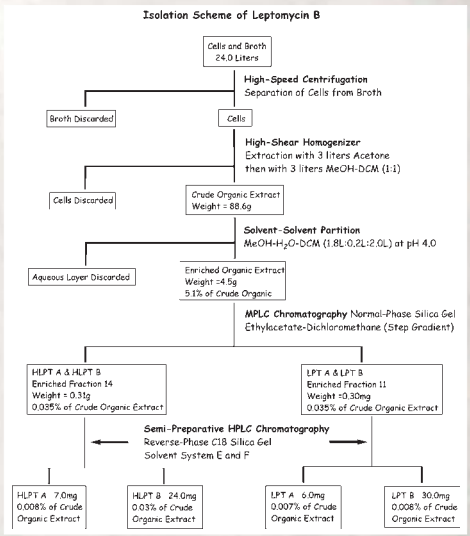
To determine whether the leptomycins were secreted into the broth, or sequestered inside the cells, the fermentation broth was filtered to separate the cells from the clarified broth and the cells were extracted with methanol. HPLC analysis revealed essentially no leptomycin in the clarified broth. It was concluded that the leptomycins, which are very non-polar and extremely water-insoluble compounds, are strongly retained inside the cell wall. The standard processing method became separation of the cells from the whole broth (i.e., 24-liter grow) by high-speed centrifugation, with the clarified broth discarded. The cell paste was homogenized with acetone, then with methanol-dichloromethane (1:1), in an OMNI high shear homogenizer. The organic extracts from the homogenized cells were collected by filtration through a Buchner funnel, combined and dried by rotary evaporation to give a crude organic extract (89.0gr).

The crude organic extract was transferred into a five-liter separatory funnel with two liters of methanol-water (10:90) and the pH adjusted to 5 with hydrochloric acid. The aqueous layer was partitioned with two liters of dichloromethane, twice. The organic layers were combined and dried by rotary evaporation to give an enriched organic extract (4.45gr, 5% of crude organic extract).

The enriched organic extract was passed through a Michel-Miller MPLC silica gel column developed with ethyl acetate-dichloromethane using a step gradient elution method. After combining fractions based on normal-phase silica gel TLC analysis, we obtained two highly enriched fractions. One fraction contained the leptomycins (fraction 11, 0.30gr, 0.34% of the crude organic extract) and the second fraction contained the hydroxyleptomycins (fraction 14, 0.30gr, 0.34% of crude organic extract).

Purification of the individual leptomycins from fractions 11 and 14 was done by preparative C18 reverse-phase HPLC. We obtained HLPT A (0.006gr, 93% purity, 0.007% of crude organic extract) and HLPT B (0.024gr, 94% purity, 0.027% of crude organic extract) from fraction 14 using solvent system E and LPT A (0.006gr, 85% purity, 0.007% of crude organic extract) and LPT B (0.030gr, 92% purity, 0.034% of crude organic extract) using solvent system F.

Figure 4. Isolation Scheme used for the purification of HLPT A, HLPT B, LPT A and LPT B from Streptomyces sp. ATCC 39366.



Structural Identification of the Leptomycins

Structural confirmation of leptomycin B was done by HPLC/MS and by comparison of experimentally acquired proton and carbon NMR data with those reported in the literature. The other three leptomycins were analyzed by HPLC/MS and identified based on their molecular ion.

Discussion

Fermentation of Streptomyces sp. ATCC39366 has been successfully carried out in large shake flask cultures to give a consistent titer of 30mg/L of leptomycin B. These compounds are found exclusively inside the cells, which allow the development of a simplified extraction and purification scheme to give reproducible batches of the four leptomycins. The method is scaleable to gram quantities to produce research grade material for anticancder research.

References

1. a) Hamamoto, T.; Gunji, S.; Tsuji, H.; Beppu, T. **Leptomycins A and B, New Antifungal Antibiotics. I. Taxonomy of the Producing Strain and their Fermentation, Purification and Characterization**. J. Antibiot. (1983), 35(6), pp639-645. b) Hamamoto, T.; Seto, H.; Beppu, T. **Leptomycins A and B, New Antifungal Antibiotics. II. Structure and Elucidation**. J. Antibiot. (1983), 35(6), pp646-605. c) Hamamoto, T.; Uozumi, T.; Beppu, T. **Leptomycins A and B, New Antifungal Antibiotics. III. Mode of Action of Leptomycin B on** Schizosaccharomyces pombe . J. Antibiot. (1985), 38(11), pp1573-1580.
2. a) Umezawa, T.; Komiyama, K.; Oka, H.; Okada, K.; Tomisaka, S.; Miyano, T.; Takano, S. **A New Antitumor Antibiotic, Kazusamycin** J. Antibiot. (1984), 37(7), pp706-711. b) Komiyama, K.; Okada, K.; Oka, H.; Tomisaka, S.; Miyano, T.; Funayama, S.; Umezawa, I. **Structural Study of a New Antitumor Antibiotic, Kazusamycin**. J. Antibiot. (1985), 38(2), pp220-223. c) Komiyama, K.; Okada, K.; Hirokawa, . ; Masuda, K.; Tomisaka, S.; Umezawa, I. **Antitumor Activity of a New Antitumor Antibiotic, Kazusamycin**. J. Antibiot. (1985), 38(2), pp224-229. d) Tunac, J.B.; Graham, B.D.; Dobson, W.E; Lenzini, M.D. **Novel Antitumor Antibiotics, CI-940 (PD 1 4, 20) and PD 4, 21** J. Antibiot., (1985), 38(4), pp460-465. e) Hurley, T.R.; Bunge, R.H.; Willmer, N.E.; Hokanson, G.C.; French, J.C. **PD 124,8 and PD 124,9 Two New Antitumor Antibiotics**. J. Antibiot. (1986), 39(12), pp1651-1656.
3. a) Hayakawa, Y.; Adachi, K.; Komeshima, N. **New Antitumor Antibiotics, Anguinomycins A and B**. J. Antibiot. (1987), 40(9), pp1349-1352. b) Hayakawa, .; Sohda, K.; Shin-Ya, K.; Hidaka, T.; Seto, H. **Anguinomycins C and D, New Antitumor Antibiotics with Selective Cytotoxicity Against Transformed Cells**. J. Antibiot. (1995), 48(9), pp954-961.
4. Abe K.; Yoshida M.; Horinouchi S.; Beppu T. **Leptolstatin from** Streptomyces sp. **SAM15 a New Gap Phase-Specific Inhibitor of the Mammalian Cell Cycle. I. Screening, Taxonomy, Purification and Biological Activities**. J. Antibiot. (1993), 46(5), pp728-734.
5. Kobayashi, M.; Higuchi, K.; Murakami, N.; Tajima, H.; Aoki, S. **Callystatin A, a Potent Cytotoxic Polyketide from the Marine Sponge,** Callyspongia truncata . Tetrahedron Lett. (1997), 38(16), pp2859-2862.
6. Schummer, D.; Gerth, K.; Reichenbach, H.; Hoefle, G. **Antibiotics from Gliding Bacterial. LXIII. Ratjadone: a New Antifungal Metabolite from** Sorangium cellulosum . Liebigs Annalen (1995), (4), pp685-688.
7. Hayakawa, .; Sohda, .; Furihata, K.; Kuzuyama, T.; Shin-Ya, K.; Seto, H. **Studies on New Antitumor Antibiotics, Leptofuranins A, B, C and D. I. Taxonomy, Fermentation, Isolation and Biological Activities**. J. Antibiot. (1996), 49(10), pp974-979.
8. a) Gorlich, D. **Transport Into and Out of the Cell Nucleus**. EMBO Journal (1998), 17(10), pp2721-2727. b) Pante, N.; Aebi, U. **Toward the Molecular Dissection of Protein Import into Nuclei**. Curr. Opin. Cell Biol. (1996), 8(3), pp397-406.
9. a) Watanabe, K.; Takizawa, N.; Katoh, M.; Hoshida, K.; Kobayashi, N.; Nagata, K. **Inhibition of Nuclear Export of Ribonucleoprotein Complexes of Influenza Virus by Leptomycin B**. Virus Research (2001), 77(1), pp31-42. b) Fukuda, M.; Asano, S.; Nakamura, T.; Adachi, M.; oshida, M.; anagida, M.; Nishida, E. **CRM1 is Responsible for Intracellular Transport Mediated by the Nuclear Export Signal**. Nature (London), (1997), 390(6657), pp308-311.
10. Roberts, B.J.; Hamelehle, K.L.; Sebolt, J.S.; Lepold, W.R. **In-vivo and In-vitro Anticancer Activity of the Structurally Novel and Highly Potent Antibiotic CI-960 and its Hydroxy Analog (PD 4, 21)**. Cancer Chemother. Pharmacol., (1986), 16, pp95-101.
11. Nettleton, D.E.; Bray, S.W.; Bush, J.A.; Bradner, W.T.; **Rigelettone Antitumor Complex**. United States Patent, 1988, USP 4,792,522 (16 pages).

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