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Hemisynthesis of Anisomycin Derivatives as Antitumor Agents

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Abstract

The antibiotic anisomycin, secreted by *Streptomyces griseolus*, can induce tumor cell death and it displays anti-metastatic activity coupled with induction of apoptosis. Herein we report the hemi-synthesis of 16 novel anisomycin derivatives and their biological activity. The protein synthesis inhibition and the effects on cancer cell proliferation and migration were assessed for two series of molecules to determine structure-activity relationships. The secondary amino group of anisomycin is essential to preserve the bioactivity. Although, the natural product is the most active component of the series but an active derivative has been identified.

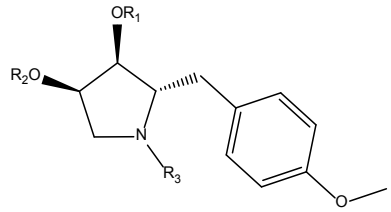
Keywords: Anisomycin; Cancer; Pyrrolidine; Tumor cell death; Anti-metastatic activity

Introduction

The pyrrolidine antibiotic anisomycin (ANS, also known as flagecidin), produced by *Streptomyces griseolus*, was first described in the mid-1950s as an anti-parasitic agent: active against several pathogenic protozoa and fungi [1]. Its structure and stereochemistry was solved in 1965 [2]. It has been used in Humans as a treatment for amoebic dysentery and trichomonas vaginitis. It has also been shown that it has antiviral and antitumor activities by means via apoptosis as well as immunosuppressive activity which is superior to cyclosporine A [3]. ANS is commonly referred as a ribotoxic stress inducer - via ribosome binding, and inhibition of peptidyl transferase activity - and as a reversible blocker of protein synthesis. This natural product has been extensively used in studies about learning, memory and synaptic plasticity [4]. ANS activates the stress-activated kinase (SAPK) pathways but multiple mechanisms are indeed implicated in its mode of action and this drug can trigger both pro- and anti-apoptotic processes in cells [5,6].

Several studies suggested that ANS can be used as an anti-tumor agent. Recently, it was shown that ANS sensitizes TRAIL-mediated hepatoma cell apoptosis via the mitochondria-associated pathway [7]. In recent *in vitro* and *in vivo* studies, ANS was shown to efficiently repress the growth of Ehrlich ascites carcinoma cells through caspase signaling, with an effect superior to the one of anticancer drug Adriamycin [8]. ANS-induced cancer cell death is well documented [9-12]. Moreover, after a chemical screening ANS was identified as an anoikis sensitizer that acts by decreasing FLIP protein synthesis with a potential link to metastasis [13]. In a recent paper [14], anisomycin derivatives were shown to be as active as anisomycin, but ANS-induced cell death by these do not correlate with protein synthesis inhibition or with JNK activation, indicating an alternative mechanistic pathway for these derivatives. These observations prompted us to investigate the anticancer activity of ANS and its derivatives, especially because a recent toxicology study indicated that ANS has no significant side effects at effective therapeutic doses [15].

Several synthesis of ANS have been described. Much attention has been focused toward the development of convenient routes to ANS derivatives [16-21]. Even if, in most cases, the ANS analogues were found to be much less active than the parent natural product. For example a study developed by Goard et al. [22] has shown that amide or carbamate derivatives of anisomycin are less active. Herein we report the synthesis of 18 derivatives of ANS (Table 1), obtained through modification of the natural product, and their biological characterization. Two series of molecules were designed and prepared. The first series of compounds (1-10) refers to derivatives bearing acetyl



Compound	R_1	R_2	R_3
Anisomycin	$COCH_3$	H	H
1	H	H	H
2	$COCH_3$	H	$COCH_3$
3	$COCH_3$	$COCH_3$	$COCH_3$
4	H	H	$COCH_3$
5	$COCH_3$	H	COC_6H_5
6	$COCH_3$	COC_6H_5	COC_6H_5
7	$COCH_3$	H	COC_6H_5-4OCH_3
8	$COCH_3$	COC_6H_5-4OCH_3	COC_6H_5-4OCH_3
9	$COCH_3$	H	$CH_2C_6H_5$
10	$COCH_3$	H	$COOC(CH_3)_3$
11	H	$COCH_3$	$COOC(CH_3)_3$
16	H	H	$COOC(CH_3)_3$
18	$COCH_3$	$CH_2C_6H_5$	H
19	H	$CH_2C_6H_5$	H.HCl
20	H	COC_6H_5-4OCH_3	H.HCl
21	H	COC_6H_5	H.HCl
22	H	$COCH_3$	H.HCl
23	CH_3	H	H.HCl

Table 1: Chemical structures of ANS derivatives.

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and/or benzoyl substituents (Scheme 1). The second series (18-23) corresponds to molecules that preserve the secondary amino group of ANS (Scheme 2).

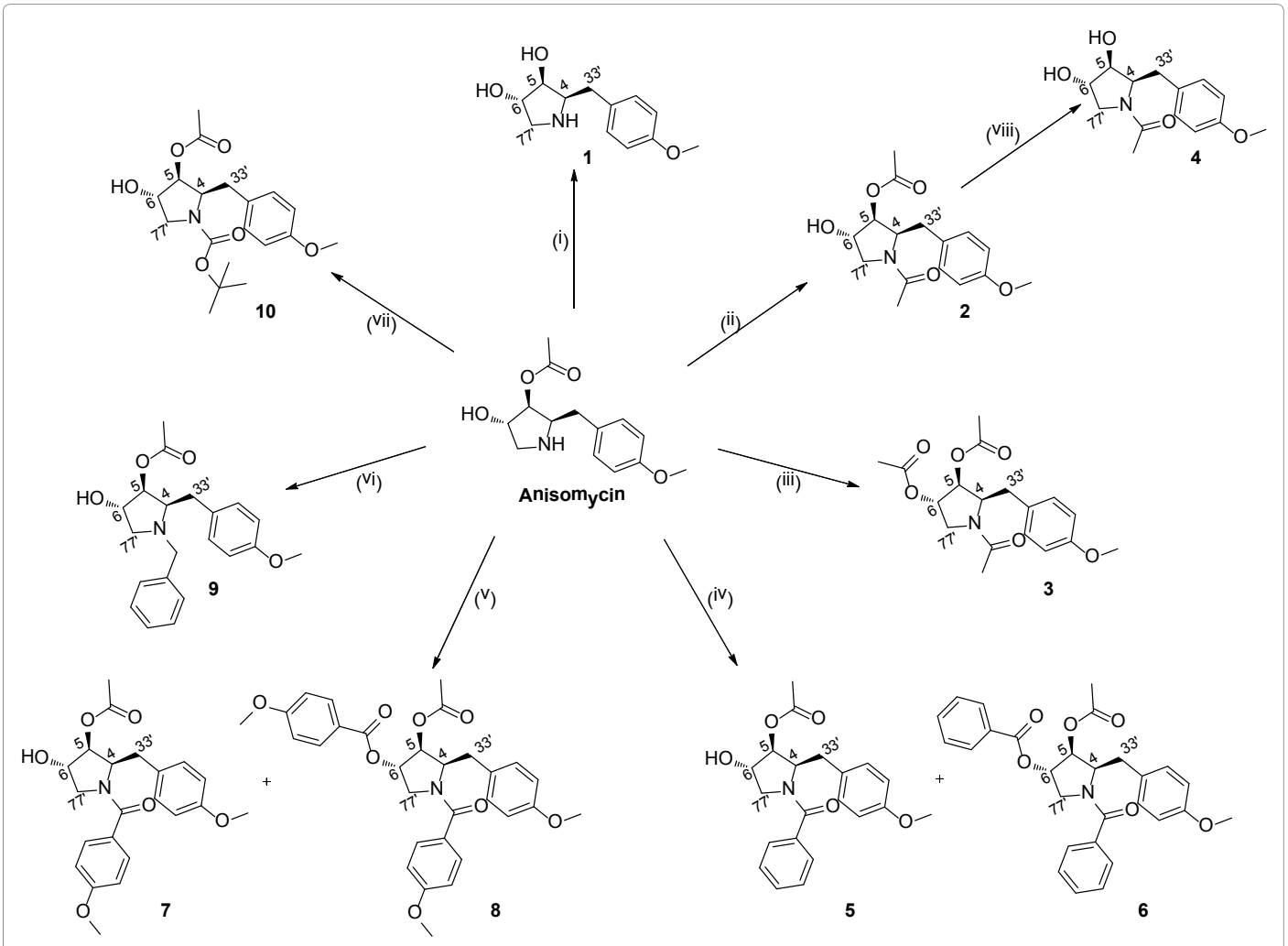
All compounds are indicated in Table 1. The synthetic pathways to obtain the ANS derivatives 1-10 are outlined in Scheme 1. ANS (commercially available) was hydrolyzed with sodium hydroxide to give deacetylanisomycin 1 previously described by Beereboom et al. [2] ANS was acetylated with acetic anhydride to give compound 2 and basic hydrolysis of the *N*-acetylanisomycin gave 4. Compound 3 was synthesized under peptide coupling conditions (EDC.HCl/HOBt) in dry THF. The acylation of ANS with acyl chloride or alkylation with benzyl bromide in the presence of base (K_2CO_3 or NEt_3) gave mono-substituted compounds 5, 7 and 9 and di-substituted compounds 6 and 8. NMR analysis showed that N-acylated pyrrolidine 2, 3, 4, 5, 6, existed as two rotamers. We performed a study to acquire variable temperature NMR data for compounds that are present as rotameric mixtures but a better assignment of the NMR spectra could not be made. To facilitate the synthesis of compounds 12, 14 and 15, the amino group of ANS was protected with Boc anhydride in a good yield to give compound 10.

The synthesis of compounds 12-23 is illustrated in Scheme 2. Boc-protected compound 10 reacted with two acyl chloride or benzyl bromide in the presence of a base (K_2CO_3 or NEt_3) to give compounds 12, 13 and 14. The side product 11 was obtained by a transesterification of the alcohol group. Compound 15 was synthesized under peptide coupling conditions (EDC.HCl/HOBt) in dry THF. Treatment of compound 10 with methyl iodide in the presence of NaH provided 17. The position of the hydroxyl group on the ring of compound 11 and 17 was determined by ^1H deuteriated NMR studies and ^2D NMR analysis. Product 16 was obtained via the deacetylation of compound 10 under basic conditions. Deprotection of corresponding carbamate 12, 13, 14 and 15 with HCl / isopropanol provided compound 18 and deacetylated compounds 19, 20, 21 and 22, in the form of hydrochloride salts. Compound 17 was deprotected with the same method to give hydrochloride compound 23.

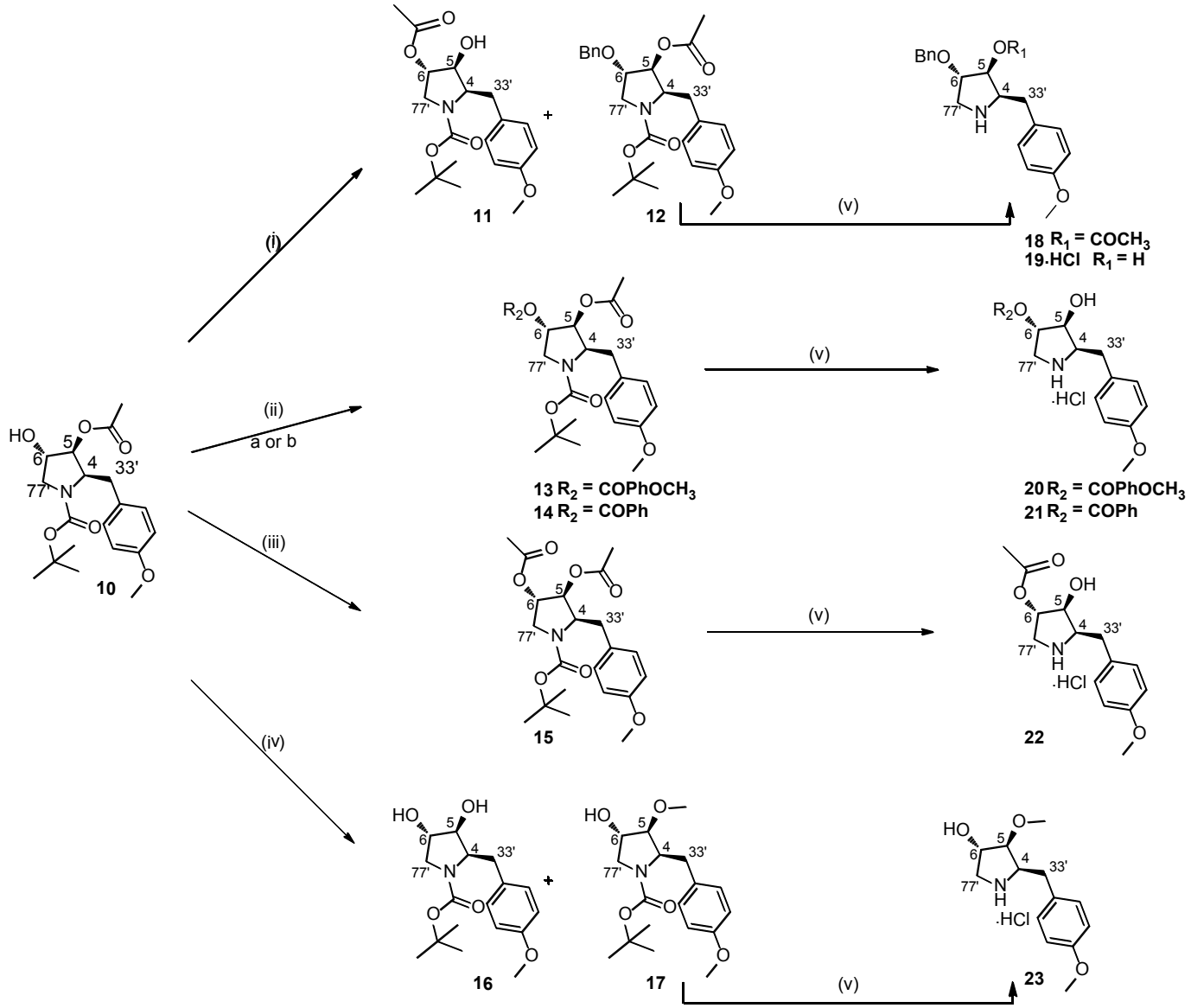
Experimental Section

Biological assays and methods

Cellular viability: The A549, HCT116, Namalwa, A375 and MDA-MB-231 cell lines were obtained from the ATCC (LGC PromoChem); The SNB-78 cell line was provided by the National Cancer Institute (NCI-0503738). A549 and HCT116 cells were cultured in MEM medium (Sigma) supplemented with 5 % fetal bovine serum (FBS), Namalwa



Scheme 1: Reagents and conditions: (i) NaOH 1 N, reflux, 2 h; (ii) acetic anhydride, rt, 24 h; (iii) DIEA, HOBt.H_2O, EDC.HCl, acetic acid, dry THF, rt, 18 h; (iv) benzoyl chloride, K_2CO_3, acetone, reflux, 3 h; (v) *p*-anisoyl chloride, NEt_3, dry CH_2Cl_2, reflux, 2 h; (vi) benzyl bromide, K_2CO_3, acetone, reflux ,4 h; (vii) DIEA, $(Boc)_2O$, 1,4-dioxane, water, rt, 24 h; (viii) (a) MeOH, NH_4OH, reflux, 15 min; (b) NH_4OH, rt, 24 h.



Scheme 2: Reagents and conditions: (i) benzyl bromide, K_2CO_3, acetone, reflux, 7 h; (ii) (a) *p*-anisoyl chloride or (b) benzoyl chloride, K_2CO_3, acetone, reflux, 7 h; (iii) DIEA, HOBt.H_2O, EDC.HCl, acetic acid, dry THF, rt, 18 h; (iv) Methyl iodide, NaH, dry THF, rt, 72 h; (v) HCl/isopropanol [5-6 N], rt, 2 h.

cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10 % FBS, A-375 cells were cultured in DMEM (Sigma) supplemented with 10 % FBS. MDA-MB-231 cells were cultured in DMEM medium (Sigma) with 10 % FBS, SNB78 in RPMI 1640 with 10 % FBS. All media were supplemented with 2 mM Glutamine (Sigma), 100 µg/mL and 1.25 µg/mL Fungizone (Gibco). Proliferation assays were performed as previously described [23]

Protein synthesis inhibition: The cell line DLD1-Luc-4Ub was engineered as previously described [23]. Briefly, cells were seeded at 2x104 cells/mL in 96-well plates (100 µl/well). Cells were treated for 1 hour with the test drug and protein synthesis was evaluated via measurement of the luciferase activity (Luciferase Assay Reagent #E1501 Promega) with a luminometer.

Western blotting analysis: MDA-MB-231 cells were treated for 4 hours with the test compound at different concentrations, then washed with PBS, lysed with RIPA and pelleted by centrifugation (14,000 rpm, 10 min). The total protein level was recorded by using the Bradford method; volume of supernatant corresponding to 80 µg soluble proteins was deposited on SDS-PAGE (10-12 % acrylamide depending on the cyclin-D1 or c-myc analysis). For cyclin-D1, rabbit anti-cyclin-D1 antibody (Cell Signaling #9402) or rabbit anti-c-Myc antibody (Cell Signaling #2922) were used at a 1:1000 dilution. Dots were visualized by chemoluminescence by using goat anti-rabbit antibody peroxidise-conjugate at 1:2000 dilution (Jackson ImmunoResearch #111-035-144). Protein quantification was measured by using a Fluor S-Multilmager (Biorad).

Antimigratory activity: Inhibition of cell migration was performed in Boyden chambers (BD Falcon HTS Fluoroblock 96-Multiwell-inset System 8µm pore diameter #351164).26 MDA-MB-231 or SNB-78 cells were seeded in their corresponding medium (50 µL) at 5×10^5 or 3×10^5 cells/mL (no FBS), respectively in the upper chamber, in the absence or presence of different concentrations of the test compound. To activate migration of cells through the separative membrane, the

lower chamber was filled with culture medium containing 10 % FBS. For basal migration, the upper chamber with cells and lower chamber without cells were filled with medium without FBS. 24 hours later, the medium of the two chambers was discarded and Calcein-AM 2 µM was added for cell labelling during 1 hour at 37°C. The measurement of cell density was recorded with a spectrofluorometer (EnVision PerkinElmer); Calcein-AM was excited at 492 nm and the fluorescence emission intensity was recorded at 517 nm.

General chemistry

Anisomycin was purchased from Chem-Impex. All commercial reagents and solvents were used without further purification. All reactions were monitored by analytical thin-layer chromatography (TLC) on 0.2 mm, Polygram SIL G/UV254 plates (Macherey-Nagel); compounds were visualized by UV (254 and 366 nm) and/or with iodine. Flash chromatography (FC) was performed with silica gel Kieselgel Si 60 0,015-0,040 mm (Macherey-Nagel). Melting points (Mp) were determined with a Büchi 535 capillary melting point apparatus and remain uncorrected. The structures of each compound were supported by IR (neat, FT- BrückerAlpha instrument) and by 1H and 13C NMR at 300 MHz and 75 MHz respectively on a Brucker DPX−300 spectrometer. Optical rotations were measured at 20°C on a Perkin-Elmer 343 polarimeter. Specific rotations were recorded in methanol (c=1,0 mg/mL). Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (TMS), J values are in Hertz (Hz), and the splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet; bs, broad singlet. The purity of compounds was checked using LC-MS system Thermo Electron Surveyor MSQ. The mass spectra were operated in the atmospheric pressure chemical ionization mode ($APCI^+$). HRMS experiments were performed on Q ExactiveBenchtop LC-MS/MS (Thermo Scientific). Experimental analysis as 13C NMR and mass spectroscopy data was described for a few of the representative compounds.

Synthesis of (2R,3S,4S)-2-(4-methoxybenzyl)pyrrolidine-3,4-diol (1): Anisomycin (0.15 g, 0.56 mmol) and 6 mL of 1 N sodium hydroxide was refluxed for 2 hours and filtered while hot. The filtrate was cooled 24 hours at 0°C. Precipitated solid was collected by filtration and washed with cold water (10 mL) and diethyl ether (10 mL) to give (**1**) as white solid (74%). Mp: 175-177 °C. IR (cm^{-1}: neat): 3351 (OH), 3270 (OH), 2915 (NH), 1245 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.15 (d, 2H, *J* = 8.4 Hz, ArH), 6.81 (s, 2H, *J* = 8.4 Hz, ArH), 4.61 (d, 1H, *J* = 2.9 Hz, OH), 4.55 (d, 1H, *J* = 4.7 Hz, OH), 3.83 (m, 1H, H-5), 3.70 (s, 3H, OCH_3), 3.47 (m, 1H, H-6), 3.15 (dd, 1H, *J* = 11.3 and 5.7 Hz, H-7), 2.97 (m, 1H, H-4) , 2.70 (dd, 1H, *J* = 13.2 and 7.3 Hz, H-3), 2.54 (d, 1H, *J* = 7.0 Hz, H-3'), 2.37 (dd, 1H, *J* = 11.8 and 2.1 Hz, H-7'), 1.92 (s, 1H, NH). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{12}H_{17}NO_3$, m/z: 224. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{12}H_{17}NO_3$, m/z: 224.1281 found m/z: 224.1276. $[\alpha]^{20}_D$ -22°, lit2.

Synthesis of (2R,3S,4S)-1-acetyl-4-hydroxy-2-(4-methoxybenzyl) pyrrolidin-3-yl acetate (2): Anisomycin (0.60 g, 2.26 mmol) and 18 mL of acetic anhydride was stirred at room temperature for 24 hours. Precipitated solid was collected by filtration and washed with water (10 mL) and diethylether (10 mL). Crystallisation from EtOH gave pure (2) as white crystals (65%). Mp: 185-187 °C. IR (cm^{-1}: neat): 3254 (OH), 1740 (CO), 1605 (NCO), 1228 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.07 (m, 2H, ArH), 6.84 (m, 2H, ArH), 5.47 (d, 0.7H, J = 4.3Hz, OH), 5.41 (d, 0.3H, J = 6.1 Hz, OH), 4.81 (t, 0.3H, J = 6.0 Hz, H-5), 4.65 (t, 0.7H, J = 6.0Hz, H-5), 4.30 (m, 1H, H-6), 4.12 (m, 0.3H, H-4), 3.88 (m, 0.7H, H-4), 3.53 (dd, 0.7H, J = 7.9 and 11.6 Hz, H-7), 3.40 (m, 0.3H, H-3'), 3.27 (m, 0.7H, H-3), 3.20 (m, 0.3H, H-3), 3.12 (dd, 0.7H, *J* = 6.1 and 14.0 Hz, H-3'), 2.75 (dd, 0.3H, *J* = 6.1 Hz and 12.5 Hz, H-7), 2.63 (dd, 1H, *J* = 10.3 and 13.4 Hz, H-7'), 2.03 (s, 0,9H, CH_3), 2.01(s, 2.1H, CH_3), 1.97 (s, 2.2H, CH_3), 1.59 (s, 0.8H, CH_3). ^{13}C NMR (DMSO–*d6*) δ ppm: 21.09, 21.43, 23.20, 32.42, 34.45, 49.66, 52.85, 55.46, 58.60, 59.49, 70.14, 70.69, 77.22, 78.94, 114.11, 129.84, 130.40, 130.69, 130.99, 158.03, 158.37, 169.22, 169.95, 170.08, 170.44. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{16}H_{21}NO_5$, m/z: 308, 248 (M^+ -CH_3COOH). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{16}H_{21}NO_5$, m/z: 308.1493, found m/z: 308.1486. [α]20D +42.5°, lit2.

Synthesis of (2R,3S,4S)-1-acetyl-2-(4-methoxybenzyl) pyrrolidine-3,4-diyl diacetate (3): *N,N*-diisopropylethylamine (2.35 mL, 13.59 mmol), EDC.HCl (1.45 g, 7.54 mmol) and $HOBt.H_2O$ (0.69 g, 4.53 mmol) were added to a solution of acid acetic (0.19 mL, 3.32 mmol) in 10 mL of dry THF. After 30 min, anisomycin (0.40 g, 1.51 mmol) was added and the mixture was stirred at room temperature. After 18 hours, the solvent was removed under reduced pressure. The residue was dissolved in AcOEt and washed with solution of HCl [1 N] (3 x 20 mL), 10 % solution of $NaHCO_3$ (3×20 mL), brine and dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/ MeOH 9:1) to give (3) as a yellow oil (30%). IR (cm^{-1}: neat): 1737 (CO), 1647 (NCO), 1219 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.08 (d, 2H, *J* = 7.8 Hz, ArH), 6.85 (m, 2H, ArH), 5.21 (t, 0.4H, *J* = 6.6 Hz, H-5), 5.10 (m, 0.4H, H-6), 4.96 (t, 0.6H, *J* = 6.0 Hz, H-5), 4.87 (m, 0.6H, H-6), 4.40 (m, 1H, H-4), 3.71 (s, 3H, OCH_3), 3.63 (dd, 0.5H, *J* = 6.9 Hz, H-7 and H-7'), 3.39 (m, 1.4H, H-3 and H-3'), 3.02 (dd, 0.6H, *J* = 3.8 and 13.8 Hz, H-3 and H-3'), 2.70 (m, 1.5H, H-7 and H-7'), 2.08 (s, 0.2H, CH_3), 2.05 (s, 1.2H, CH_3), 2.01 (s, 2.8H, CH_3), 1.99 (s, 1.8H, CH_3), 1.96 (s, 1.8H, CH_3), 1.46 (s, 1.2H, CH_3). ^{13}C NMR (DMSO–*d6*) δ ppm: 20.97, 21.15, 22.91, 32.43, 33.88, 47.13, 50.00, 55.40, 57.94, 59.44, 73.46, 74.11, 74.34, 76.02, 114.17, 114.27, 129.53, 130.13, 130.54, 131.14, 158.14, 158.49, 168.96, 169.69, 169.90, 170.19, 170.21, 170.62. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{18}H_{23}NO_6$, m/z: 350, 308 (M^+ -CH_3CHO). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{18}H_{23}NO_6$, m/z: 350.1598, found m/z: 350.1593.$[\alpha]^{20}_D$ +86.9°.

Synthesis of 1-[(2R,3S,4S)-3,4-dihydroxy-2-(4-methoxybenzyl) pyrrolidin-1-yl]ethanone (4): *N*-acetylanisomycin 2 (0.20 g, 0.65 mmol) was suspended in 5 mL of methanol. Concentrated ammonium hydroxide (1 mL) was added and the suspension was refluxed to give a clear solution. Concentrated ammonium hydroxide (0.7 mL) was added and the solution was stirred at room temperature. After 24 hours, the solvent was removed under pressure to give oil that was crystallized from ethyl acetate/cyclohexane and filtered to give pure (4) as white crystals (81%). Mp: 139-140 °C. IR (cm^{-1}: neat): 3367 (OH), 3154 (OH), 1596 (NCO), 1250 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.17 (d, 1.3H, *J* = 8.2 Hz, ArH), 7.08 (d, 0.7H, *J* = 8.4 Hz, ArH), 6.82 (m, 2H, ArH), 5.43 (d, 0.4H, *J* = 3.8 Hz, OH), 5.20 (d, 0.6H, *J* = 4.3 Hz, OH), 5.06 (d, 0.4H, *J* = 4.8 Hz, OH), 4.99 (d, 0.6H, *J* = 2.9 Hz, OH), 3.90 (m, 2H, H-5 and H-6), 3.70 (s, 3H, OCH_3), 3.66 (m, 1H, H-4), 3.50 (dd, 0.7H, *J* = 4.1 and 10.9 Hz, H-3'), 3.40 (dd, 0.7H, *J* = 6.7 and 12.7 Hz, H-7'), 3.19 (m, 0.9H, H-3' and H-3 and H-7'), 3.12 (dd, 0.7H, *J* = 2.8 and 13.2 Hz, H-3), 2.94 (dd, 0.3H, *J* = 4.8 and 13.4 Hz, H-7), 2.76 (dd, 0.7H, *J* = 9.4 and 12.5 Hz, H-7), 1.93 (s, 1.9H, CH_3), 1.41 (s, 1.1H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{14}H_{19}NO_4$, m/z: 266. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{14}H_{19}NO_4$, m/z: 266.1387, found m/z: 266.1381. $[\alpha]^{20}_D$ +45.4°.

Synthesis of (2R,3S,4S)-1-benzoyl-4-hydroxy-2-(4-methoxybenzyl) pyrrolidin-3-yl acetate (5) and (2R,3S,4S)-1-benzoyl-4-benzoate-2-(4-methoxybenzyl)pyrrolidin-3-yl acetate (6): A solution of anisomycin (0.20 g, 0.75 mmol), benzoyl chloride (0.12 mL, 1.06 mmol) and potassium carbonate (0.41 g, 3.00 mmol) in acetone (10 mL) was refluxed for 3 hours and was filtered. The filtrate was concentrated in vacuo and the two compounds were separated by flash chromatography on silica gel (CH_2Cl_2/

MeOH 95:05) to give after precipitated with diethylether (20 mL), title compounds (5) as white solid (47%) and (6) as white solid (6%).

Compound (5): Mp: 173-174 °C. IR (cm^{-1}: neat): 3190 (OH), 1737 (CO), 1604 (NCO), 1232 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.51 (m, 4.7H, ArH), 7.30 (m, 0.3H, ArH), 7.16 (d, 1.8H, *J* = 7.6 Hz, ArH), 6.87 (d, 1.8H, *J* = 7.6 Hz, ArH), 6.69 (m, 0.4H, ArH), 5.56 (m, 0.1H, OH), 5.41 (d, 0.9H, OH), 4.78 (m, 0.1H, H-5), 4.71 (m, 0.9H, H-5), 4.51 (m, 0.9H, H-6), 4.38 (m, 0.1H, H-6), 4.13 (m, 0.1H, H-4), 3.84 (m, 0.9H, H-4), 3.76 (m, 1H, H-3'), 3.72 (s, 3H, OCH_3), 3.34 (m, 1H, H-7'), 3.17 (d, 1H, *J* = 10.3 Hz, H-3), 2.74 (m, 1H, H-7), 2.12 (s, 2.6H, CH_3), 1.98 (s, 0.4H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{21}H_{23}NO_5$, m/z: 370. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{21}H_{23}NO_5$, m/z: 370.1649, found m/z: 370.1644. $[\alpha]^{20}_D$ -66.5°.

Compound (6): Mp: 147-148°C. IR (cm^{-1}: neat): 1734 (CO), 1712 (CO), 1636 (NCO), 1230 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm 8.00: (m, 0.5H, ArH), 7.85 (m, 1.5H, ArH), 7.66 (m, 1H, ArH), 7.50 (m, 6H, ArH), 7.22 (d, 1.6H, *J* = 6.2 Hz, ArH), 6.86 (d, 1.6H, *J* = 7.6 Hz, ArH), 6.75 (m, 0.8H, ArH), 5.44 (m, 0.5H, H-5), 5.17 (m, 1.5H, H-5 and H-4), 4.78 (m, 0.8H, H-6), 4.54 (m, 0.2H, H-6), 4.03 (m, 1H, H-3'), 3.72 (s, 3H, OCH_3), 3.43 (m, 1H, H-7'), 3.25 (m, 1H, H-3), 2.87 (m, 1H, H-7), 2.13 (s, 2.3H, CH_3), 2.01 (m, 0.7H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{28}H_{27}NO_6$, m/z: 474. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{28}H_{27}NO_6$, m/z: 474.1911, found m/z: 474.1901.

Synthesis of (2R,3S,4S)-1-(4-methoxybenzoyl)-4-hydroxy-2-(4-methoxybenzyl)pyrrolidin-3-yl acetate (7) and (2R,3S,4S)-1-(4-methoxybenzoyl)-4-(4-methoxybenzoate)-2-(4-methoxybenzyl) pyrrolidin-3-yl acetate (8): A solution of anisomycin (0.30 g, 1.13 mmol), p-anisoyl chloride (0.30 mL, 2.26 mmol) and triethylamine (0.47 mL, 3.39 mmol) in dry CH_2Cl_2 (5 mL) was refluxed for 2 hours. The organic layer was washed with water (15 mL), solution of HCl [1 N] (15 mL), brine and dried over MgSO4. The solvent was removed under reduced pressure and the residue was separated by flash chromatography on silica gel (CH_2Cl_2/AcOEt7: 3) to give two compounds 7 and 8. Crystallization from EtOH gave pure (**7**) as white crystals (18%). Compound (8) was washed with petroleum ether to give beige solid (12%).

Compound (7): Mp: 193-194 °C. IR (cm^{-1}: neat): 3365 (OH), 1735 (CO), 1601 (NCO), 1234 (ArOCH3). ^1H NMR (DMSO–*d6*) δ ppm: 7.56 (d, 2H, J = 7.2 Hz, ArH), 7.14 (m, 2H, ArH), 6.99 (d, 2H, J = 8.5 Hz, ArH), 6.86 (m, 2H, ArH), 5.39 (m, 1H, OH), 4.70 (m, 1H, H-5), 4.49 (m, 1H, H-6), 3.84 (m, 2H, H-3 and H-4), 3.81 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.25 (m, 2H, H-7 and H-3'), 2.70 (m, 1H, H-7'), 2.11 (s, 3H, CH_3). ^{13}C NMR (DMSO–*d6*) δ ppm: 21.27, 32.02, 55.36, 55.75, 56.89, 60.44, 71.50, 76.50, 113.97, 114.26, 128.79, 130.16, 130.69, 158.11, 161.35, 169.94, 170.58. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{22}H_{25}NO_6$, m/z: 400, 340 (M^+ -CH_3COOH), 322 (M^+ -CH_3COOH -H_2O). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{22}H_{25}NO_6$, m/z: 400.1755, found m/z: 400.1751.

Compound (8): Mp: 109-110°C. IR (cm^{-1}: neat): 1746 (CO), 1714 (CO), 1604 (NCO), 1248 (ArOCH3). ^{1}HNMR(DMSO–*d6*) δ ppm: 7.81 (m, 2H, ArH), 7.51 (m, 2H, ArH), 7.19 (m, 2H, ArH), 7.03 (d, 2H, *J* = 8.9 Hz, ArH), 6.96 (d, 2H, *J* = 6.9 Hz, ArH), 6.84 (m, 2H, ArH), 5.12 (m, 2H, H5 and H-6), 4.72 (m, 1H, H-4), 4.10 (m, 1.3H, H-7 and H-3), 3.82 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.71 (m, 1H, OCH_3), 3.48 (m, 1.2H, H-3 and H-3'), 2.78 (m, 1.1H, H-7' and H-3'), 2.51 (m, 0.4H, H-7'), 2.13 (s, 3H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{30}H_{31}NO_8$, m/z: 534. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{30}H_{31}NO_8$, m/z: 534.2122, found m/z: 534.2119.

Synthesis of (2R,3S,4S)-1-benzyl-4-hydroxy-2-(4-methoxybenzyl) pyrrolidin-3-yl acetate (9): A solution of anisomycin (0.30 g, 1.13 mmol), benzyl bromide (0.19 mL, 1.58 mmol) and potassium carbonate (0.63 g, 4.52 mmol) in acetone (10 mL) was refluxed for 4 hours and was filtered. The filtrate was concentrated in vacuo and the residue was separated by flash chromatography on silica gel (CH_2Cl_2/MeOH 95:05) to give after precipitated with petroleum ether (10 mL), compound (9) as beige solid (16%). Mp: 73-74 °C. IR (cm^{-1}: neat): 3245 (OH), 1733 (CO), 1241 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.26 (m, 5H, ArH), 7.09 (d, 2H, *J* = 8.5 Hz, ArH), 6.83 (d, 2H, *J* = 8.5 Hz, ArH), 5.11 (d, 1H, *J* = 3.8 Hz, OH), 4.61 (m, 1H, H-6), 3.98 (m, 1H, H-5), 3.83 (m, 1H, H-4), 3.70 (s, 3H, OCH_3), 3.32 (m, 1H, H-3), 3.07 (m, 2H, CH2), 2.84 (dd, 1H, *J* = 5.0 and 12.6 Hz, H-7), 2.61 (m, 1H, H-3'), 2.12 (m, 1H, H-7'), 2.06 (s, 3H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{21}H_{25}NO_4$, m/z: 356. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{21}H_{25}NO_4$, m/z: 356.1856, found m/z: 356.1850.

Synthesis of tert-Butyl (2R,3S,4S)-3-(acetyloxy)-4-hydroxy-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (10): A solution of anisomycin (0.70 g, 2.64 mmol), *N,N*-diisopropylethylamine (0.46 mL, 2.77 mmol) and di-tert-butyl dicarbonate (0.61 g, 2.77 mmol) in 1,4-dioxane (16 mL) and water (4 mL) was stirred at room temperature. After 24 hours, the solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2, washed with water (3 x 30 mL), brine and dried over $CaCl_2$. The solvent was removed under reduced pressure. Crystallisation from EtOH gave (10) as white crystals (57%). Mp: 142-143 °C. IR (cm^{-1}: neat): 3437 (OH), 1737 (CO), 1665 (NCO), 1243 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.05 (d, 2H, *J* = 8.4 Hz, ArH), 6.84 (d, 2H, *J* = 8.4 Hz, ArH), 5.38 (d, 1H, *J* = 4.4 Hz, OH), 4.63 (m, 1H, H-5), 4.10 (m, 1H, H-6), 3.85 (m, 1H, H-4), 3.71 (s, 3H, OCH_3), 3.32 (m, 1H, H-3), 3.20 (dd, 1H, *J* = 3.8 and 11.8 Hz, H-3'), 3.13 (m, 0.4H, H-7), 3.05 (m, 0.6H, H-7), 2.67 (m, 1H, H-7'), 2.04 (s, 3H, CH_3), 1.40 (s, 9H, $(CH_3)_3$). ^{13}C NMR (DMSO–*d6*) δ ppm: 21.14, 28.49, 32.70, 33.85, 51.72, 52.71, 55.44, 59.44, 7.024, 70.54, 77.31, 77.98, 79.29, 114.15, 130.38, 130.47, 158.13, 170.14, 174.14. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{19}H_{27}NO_6$, m/z: 366, 266 (M^+ -$(CH_3)_3COOH$). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{19}H_{27}NO_6$, m/z: 366.1911, found m/z: 366.1910.

Synthesis of tert-Butyl (2R,3S,4S)-4-(acetyloxy)-3-hydroxy-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (11) and tert-Butyl (2R,3S,4S)-3-(acetyloxy)-4-(benzyloxy)-2-(4-methoxybenzyl) pyrrolidine-1-carboxylate (12): A solution of 10 (0.80 g, 2.19 mmol), benzyl bromide (0.63 mL, 5.25 mmol) and potassium carbonate (1.82 g, 13.14 mmol) in acetone (30 mL) was refluxed for 7 hours and was filtered. The filtrate was concentrated in vacuo and the residue was separated by flash chromatography on silica gel (CH_2Cl_2/AcOEt 9:1) to give two compounds, (11) as a colorless oil (9%) and (12) as a colorless oil (12%).

Compound (11): IR (cm^{-1}: neat): 3417 (OH), 1742 (CO), 1664 (NCO), 1235 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.14 (d, 2H, *J* = 8.4 Hz, ArH), 6.83 (d, 2H, *J* = 8.4 Hz, ArH), 5.67 (d, 1H, *J* = 5.6 Hz, OH), 4.65 (m, 1H, H-5), 3.93 (m, 1H, H-6), 3.80 (m, 1H, H-4), 3.70 (s, 3H, OCH_3), 3.48 (m, 1H, H-3), 3.24 (m, 1H, H-3'), 3.05 (m, 1H, H-7'), 2.85 (m, 1H, H-7), 1.96 (s, 3H, CH_3), 1.39 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{19}H_{27}NO_6$, m/z: 366. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{19}H_{27}NO_6$, m/z: 366.1911, found m/z: 366.1902.

Compound (12): IR (cm^{-1}: neat): 1743 (CO), 1690 (NCO), 1229 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.31 (m, 5H, ArH), 7.05 (d, 2H, *J* = 8.7 Hz, ArH), 6.83 (d, 2H, *J* = 8.7 Hz, ArH), 4.84 (m, 1H, H-5), 4.47 (s, 2H, CH_2), 4.14 (m, 1H, H-6), 3.90 (m, 1H, H-4), 3.71 (s, 3H, OCH_3), 3.38 (m, 2H, H-3 and H-3'), 3.02 (m, 1H, H-7'), 2.68 (m, 1H, H-7), 2.05 (s, 3H, CH_3), 1.39 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{26}H_{33}NO_6$, m/z: 456.

Synthesis of tert-Butyl (2R,3S,4S)-3-(acetyloxy)-4-(4-methoxybenzoyloxy)-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (13). A solution of 10 (0.80 g, 2.19 mmol), *p*-anisoyl chloride (0.74 mL, 5.47 mmol) and potassium carbonate (1.82 g, 13.14 mmol) in acetone (30 mL) was refluxed for 7 hours and was filtered. The filtrate was concentrated in vacuo and the residue was separated by flash chromatography on silica gel (cyclohexane/AcOEt 7:3). Crystallization from acetonitrile gave pure (13) as white crystals (30%). Mp: 94-95 °C. IR (cm^{-1}: neat): 1745 (CO), 1702 (CO), 1691 (NCO), 1245 ($ArOCH_3$), 1223 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.87 (d, 2H, *J* = 8.3 Hz, ArH), 7.11 (d, 2H, *J* = 8.8 Hz, ArH), 7.03 (d, 2H, *J* = 8.3 Hz, ArH), 6.86 (d, 2H, *J* = 8.8 Hz, ArH), 5.07 (s, 2H, H-6 and H-5), 4.33 (m, 1H, H-4), 3.82 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.61 (dd, 1H, *J* = 5.7 and 12.4 Hz, H-3), 3.42 (m, 1H, H-3'), 2.96 (m, 1H, H-7), 2.79 (dd, 1H, *J* = 8.3 and 12.9 Hz, H-7'), 2.07 (s, 3H, CH_3), 1.35 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{27}H_{33}NO_8$, m/z: 500.

Synthesis of tert-Butyl (2R,3S,4S)-3-(acetyloxy)-4-(benzoyloxy)-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (14). A solution of 10 (0.80 g, 2.19 mmol), benzoyl chloride (0.64 mL, 5.47 mmol) and potassium carbonate (1.82 g, 13.14 mmol) in acetone (30 mL) was refluxed for 8 hours and was filtered. The filtrate was concentrated in vacuo and the residue was separated by flash chromatography on silica gel (cyclohexane/AcOEt 6:1) to give (14) as colorless oil (39%). IR (cm^{-1}: neat): 1747 (CO), 1722 (CO), 1692 (NCO), 1224 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.92 (d, 2H, *J* = 7.2 Hz, ArH), 7.66 (m, 1H, ArH), 7.51 (m, 2H, ArH), 7.11 (d, 2H, *J* = 8.4 Hz, ArH), 6.85 (d, 2H, *J* = 8.4 Hz, ArH), 5.10 (m, 2H, H-5 and H-6), 4.35 (m, 1H, H-4), 3.71 (s, 3H, OCH_3), 3.63 (dd, 1H, *J* = 5.5 and 12.5 Hz, H-3), 3.47 (m, 1H, H-3'), 2.92 (m, 1H, H-7), 2.79 (dd, 1H, *J* = 8.3 and 14.0 Hz, H-7'), 2.07 (s, 3H, CH_3), 1.36 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{26}H_{31}NO_7$, m/z: 470.

Synthesis of tert-Butyl (2R,3S,4S)-3,4-bis(acetyloxy)-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (15): *N,N*-diisopropylethylamine (2.04 ml, 12.33 mmol), EDC.HCl (1.31 g, 6.85 mmol) and HOBt.H_2O (0.56 g, 4.11 mmol) were added to a solution of acid acetic (0.17 mL, 3.01 mmol) in 20 mL of dry THF. After 30 minutes, compound (10) (0. 50 g, 1.37 mmol) was added and the mixture was stirred at room temperature. After 18 hours, the solvent was removed under reduced pressure. The residue was dissolved in AcOEt and washed with solution of HCl [1 N] (3 x 20 mL), 10 % solution of $NaHCO_3$ (3 x 20 mL), brine and dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (cyclohexane/AcOEt 7:3) to give (15) as a colorless oil (59%). IR (cm^{-1}: neat): 1742 (CO), 1691 (NCO), 1217 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.06 (d, 2H, *J* = 8.6 Hz, ArH), 6.82 (m, 2H, *J* = 8.6 Hz, ArH), 4.95 (m, 1H, H-5), 4.77 (m, 1H, H-6), 4.21 (m, 1H, H-4), 3.71 (s, 3H, OCH_3), 3.52 (dd, 1H, *J* = 6.5 and 12.5 Hz, H-3), 3.28 (m, 1H, H-3'), 2.90 (m, 1H, H-7), 2.72 (dd, 1H, *J* = 8.1 and 13.4, Hz, H-7'), 2.05 (m, 3H, CH_3), 1.99 (s, 3H, CH_3), 1.35 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{21}H_{29}NO_7$, m/z: 408.

Synthesis of tert-Butyl (2R,3S,4S)-3,4-dihydroxy-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (16) and tert-Butyl (2R,3S,4S)-4-hydroxy-3-methoxy-2-(4-methoxybenzyl)pyrrolidine-1-carboxylate (17): A solution of 10 (0.44 g, 1.20 mmol), methyl iodide (0.08 mL, 1.32 mmol) and sodium hydride, 60% (0.05 g, 1.20 mmol) in dry THF (10 mL) was stirred for 72 hours. The mixture was neutralized by HCl [1 N] and extracted with AcOEt (3 x 15 mL). The organic layer was washed with water, brine and dried over $MgSO_4$. The solvent was removed under reduced pressure.The residue was purified by flash chromatography on silica gel (cyclohexane/AcOEt 9:1) to give two compounds, (17) as a colorless oil (12%) and, after precipitated with diethyl ether, (16) as a beige solid (16%).

Compound (16): Mp: 135-136 °C. IR (cm^{-1}: neat): 3425 (OH), 2915 (OH), 1649 (NCO), 1239 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.15 (d, 2H, *J* = 8.6 Hz, ArH), 6.82 (d, 2H, *J* = 8.6 Hz, ArH), 5.17 (d, 1H, *J* = 10.9 Hz, OH), 4.92 (m, 1H, OH), 3.73 (m, 2H, H-6 and H-5), 3.71 (s, 3H, OCH_3), 3.64 (m, 1H, H-4), 3.17 (m, 2H, H-3 and H-3'), 2.81 (m, 2H, H-7' and H-7), 1.40 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{17}H_{25}NO_5$, m/z: 324. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{17}H_{25}NO_5$, m/z: 324.1806, found m/z: 324.1796.

Compound (17): IR (cm^{-1}: neat): 3418 (OH) 3353 (OH), 1661 (NCO), 1243 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 7.14 (d, 2H, *J* = 7.7 Hz, ArH), 6.82 (d, 2H, *J* = 7.7 Hz, ArH), 5.33 (m, 1H, OH), 3.81 (m, 1H, H-5), 3.73 (m, 1H, H-6), 3.71 (s, 3H, OCH_3), 3.49 (m, 2H, H-3 and H-3'), 3.33 (m, 1H, H-4), 3.18 (s, 3H, OCH_3), 2.92 (m, 2H, H-7' and H-7), 1.37 (s, 9H, $(CH_3)_3$). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{18}H_{27}NO_5$, m/z: 338.

Synthesis of (2R,3S,4S)-4-(benzyloxy)-2-(4-methoxybenzyl) pyrrolidin-3-yl acetate (18) and (3S,4S,5R)-4-hydroxy-5-(4-methoxybenzyl)pyrrolidin-3-yl benzoate hydrochloride (19): Compound (**12**) (0.19 g, 0.41 mmol) and 7 mL of HCl/isopropanol [5-6 N] was stirred at room temperature. After 2 hours, the solvent was removed under reduced pressure. The residue was separated by flash chromatography on silica gel (CH_2Cl_2/MeOH 9:1) to give two compounds, (18) as a yellow oil (40%) and crystallization from acetonitrile gave pure (19).HCl as white crystals (18%).

Compound (18): IR (cm^{-1}: neat): 2930 (alkyl pyrrolidin), 1733 (CO), 1237 (ArOCH3). ^1H NMR (DMSO–*d6*) δ ppm: 7.30 (m, 5H, ArH), 7.03 (d, 2H, *J* = 8.2 Hz, ArH), 6.82 (d, 2H, *J* = 8.2 Hz, ArH), 4.85 (m, 1H, H-5), 4.51 (m, 2H, CH_2), 3.87 (m, 1H, H-6), 3.71 (s, 3H, OCH_3), 3.25 (m, 2H, H-3' and H-4), 2.66 (m, 3H, H-7' and H-7 and H-3), 2.08 (s, 3H, CH_3). ^{13}C NMR (DMSO–*d6*) δ ppm: 21.45, 32.99, 34.27, 50.40, 51.54, 55.51, 62.09, 70.73, 71.08, 74.20, 78.30, 84.25, 114.13, 127.88, 128.77, 130.19, 130.49, 131.82, 138.67, 158.02, 170.39. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{21}H_{25}NO_4$, m/z: 356, 266 (M^+ -$CH_3C_6H_5$). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{21}H_{25}NO_4$, m/z: 356.1856, found m/z: 356.1852.

Compound (19).HCl: Mp: 178-179 °C. IR (cm^{-1}: neat): 3390 (OH), 2909 (alkyl pyrrolidin), 1245 (ArOCH3). ^1H NMR (DMSO–*d6*) δ ppm: 9.36 (s, 1H, NH_2^+), 7.30 (m, 7H, ArH), 6.90 (d, 2H, *J* = 8.0 Hz, ArH), 5.98 (m, 1H, OH), 4.51 (m, 2H, CH_2), 4.04 (m, 2H, H-5 and H-6), 3.73 (s, 3H, OCH_3), 3.52 (m, 1H, H-4), 3.58 (m, 1H, H-3'), 3.16 (m, 1H, H-3), 3.02 (m, 1H, H-7), 2.86 (m, 1H, H-7'). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{19}H_{23}NO_3$, m/z: 314. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{19}H_{23}NO_3$, m/z: 314.1751, found m/z: 314.1739.

Synthesis of (3S,4S,5R)-4-hydroxy-5-(4-methoxybenzyl) pyrrolidin-3-yl (4-methoxy)benzoate hydrochloride (20): Compound (13) (0.13 g, 0.26 mmol) and 5 mL of HCl/isopropanol [5-6 N] was stirred at room temperature. After 2 hours, the solvent was removed under reduced pressure. The residue was separated by flash chromatography on silica gel (CH_2Cl_2/MeOH 9:1) to give after precipitated with petroleum ether (5 mL) and diethyl ether (5 mL), compound (20).HCl as a beige solid (22%). Mp 132-133 °C. IR (cm^{-1}: neat): 3261 (OH), 2907 (Alkyl pyrrolidin), 1702 (CO), 1249 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 9.37 (m, 1H, NH_2^+),7.88 (d, 2H, *J* = 8.8 Hz, ArH), 7.24 (d, 2H, *J* = 8.4 Hz, ArH), 7.02 (d, 2H, *J* = 8.4 Hz, ArH), 6.85 (d, 2H, *J* = 8.8 Hz, ArH), 5.65 (m, 1H, OH), 5.13 (m, 1H, H-5), 3.97

(m, 1H, H-6), 3.82 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.54 (m, 2H, H-7 and H-4), 2.91 (m, 2H, H-3' and H-3), 2.73 (dd, 1H, *J* = 7.7 and 14.2 Hz, H-7'). ^{13}C NMR (DMSO–*d6*) δ ppm: 231.08, 48.70, 55.49, 56.03, 64.03, 72.71, 77.21, 114,49, 114.59, 121.62, 129.47, 130.69, 132.15, 158.85, 164.07, 164.71. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{20}H_{23}NO_5$, m/z: 358. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{20}H_{23}NO_5$, m/z: 358.1649, found m/z: 358.1642.

Synthesis of (3S,4S,5R)-4-hydroxy-5-(4-methoxybenzyl) pyrrolidin-3-yl benzoate hydrochloride (21): Compound (14) (0.30 g, 0.64 mmol) and 7 mL of HCl/isopropanol [5-6 N] was stirred at room temperature. After 2 hours, the solvent was removed under reduced pressure. The residue was separated by flash chromatography on silica gel (CH_2Cl_2/MeOH 9:1) to give after precipitated with acetonitrile (10 mL), compound (21.HCl as a white solid (18%). Mp > 200 °C. IR (cm^{-1}: neat): 3286 (OH), 2909 (Alkyl pyrrolidin), 1713 (CO), 1279 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 9.38 (m, 1H, NH_2^+), 7.93 (d, 2H, *J* = 8.0 Hz, ArH), 7.66 (m, 1H, ArH), 7.51 (m, 2H, ArH), 7.03 (d, 2H, *J* = 8.6 Hz, ArH), 6.84 (d, 2H, *J* = 8.0 Hz, ArH), 5.57 (m, 1H, OH), 5.14 (m, 1H, H-5), 3.96 (m, 1H, H-6), 3.71 (s, 3H, OCH_3), 3.54 (m, 2H, H-7 and H-4), 2.87 (m, 2H, H-3' and H-3), 2.70 (dd, 1H, J = 7.2 and 14.4 Hz, H-7'). ^{13}C NMR (DMSO–*d6*) δ ppm: 33.33, 50.60, 55.51, 64.03, 74.74, 80.70, 114.18, 129.19, 129.95, 130.47, 131.9074, 134.0191, 158.15, 165.46. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{19}H_{21}NO_4$, m/z: 328, 188 (M^+ -C_6H_5COOH -H_2O). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{19}H_{21}NO_4$, m/z: 328.1543, found m/z: 328.1536.

Synthesis of (3S,4S,5R)-4-hydroxy-5-(4-methoxybenzyl) pyrrolidin-3-yl acetate hydrochloride (22): Compound **(15)** (0.13 g, 0.32 mmol) and 5 mL of HCl/isopropanol [5-6 N] was stirred at room temperature. After 2 hours, the solvent was removed under reduced pressure. The residue was separated by flash chromatography on silica gel (CH_2Cl_2/MeOH 9:1) to give after precipitated with petroleum ether (5 mL), compound (**22**).HCl as a grey solid (10%) and compound 1 (40%). Mp > 200 °C. IR (cm^{-1}: neat): 3371 (OH), 2956 (NH), 2673 (Alkyl pyrrolidin), 1746 (CO), 1224 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 9.39 (m, 2H, NH_2^+), 7.22 (d, 2H, *J* = 8.5 Hz, ArH), 6.91 (d, 2H, *J* = 8.5 Hz, ArH), 6.00 (d, 1H, *J* = 3.7 Hz, OH), 4.86 (m, 1H, H-5), 4.21 (m, 1H, H-6), 3.97 (m, 1H, H-4), 3.73 (s, 3H, OCH_3), 3.43 (m, 1H, H-3'), 3.05 (m, 1H, H-3), 2.92 (m, 2H, H-7 and H-7'), 2.14 (s, 3H, CH_3). LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{19}H_{14}NO_4$, m/z: 266. HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{19}H_{14}NO_4$, m/z: 266.1387, found m/z: 266.1383.

Synthesis of (3S,4S,5R)-4-methoxy-5-(4-methoxybenzyl) pyrrolidin-3-ol hydrochloride (23): Compound (17) (0.13 g, 0.32 mmol) and 5 mL of HCl/isopropanol [5-6 N] was stirred at room temperature. After 2 hours, the solvent was removed under reduced pressure. Crystallisation from acetonitrile gave (23).HCl as white crystals (91%). Mp 185-186 °C. IR (cm^{-1}: neat): 3357 (OH), 2719 (Alkyl pyrrolidin), 1244 ($ArOCH_3$). ^1H NMR (DMSO–*d6*) δ ppm: 9.50 (m, 2H, NH_2^+), 7.26 (d, 2H, *J* = 8.7 Hz, ArH), 6.90 (d, 2H, *J* = 8.7 Hz, ArH), 5.96 (m, 1H, OH), 4.02 (m, 1H, H-5), 3.84 (m, 1H, H-6), 3.73 (s, 3H, OCH_3), 3.52 (m, 1H, H-4), 3.41 (dd, 1H, *J* = 4.8 and 13.0 Hz, H-3'), 3.25 (s, 3H, OCH_3), 3.03 (m, 2H, H-7 and H-3), 2.84 (dd, 1H, *J* = 7.6 and 14.1 Hz, H-7'). ^{13}C NMR (DMSO–*d6*) δ ppm: 30.88, 48.27, 55.50, 56.97, 63.75, 71.19, 84.0235, 114.49, 129.54, 130.54, 158.62. LCMS (APCI $(M+H)^+$ m/z) calcd for $C_{13}H_{19}NO_3$, m/z: 238, 207 (M^+ -CH_3OH). HRMS (ESI $(M+H)^+$ m/z) calcd for $C_{13}H_{19}NO_3$, m/z: 238.1438, found m/z: 238.1432.

Results and Discussion

Biochemical and cellular analyses

ANS derivatives were evaluated for protein synthesis inhibition using a DLD1-Luc-4Ub cellular assay. Human DLD-1 colon cancer cells, engineered to express a 4 ubiquitin-luciferase (DLD-1 4Ub-Luc) reporter protein [23], were treated with the respective compounds for 1 hour and protein expression was monitored 24 hours later by luminescence. As expected, ANS presents a very potent activity (EC_{50}: 32 nM) whereas the vast majority of hemi-synthetic compounds were totally inactive in this cellular assay (data not shown).

The scaffold ANS modifications as the substitution of the function of alcohol with a benzoyl or benzyl group involve a loss of activity. Incorporation of an alkyl or acyl group in *N*-position of pyrrolidine heterocycle led to a decrease of compounds activity. One molecule, compound 21, the alcohol of which is substituted by a benzoyl group displays a modest activity, 100-fold lower than that of ANS (EC_{50}: 3.2 µM). And one compound, 22, substituted by an acetyl group in position 4 of pyrrolidine explains a significant activity, with an EC_{50} of 100 nM, i.e. 3 times less than ANS (Table 2).

The dose-response curves presented in Figure 1 show that the compound 22, which only differs from ANS by the position of the acetyl group on the alcohol group (Table 1), is just slightly less active than ANS.

Afterwards, we investigated the cytotoxic properties of the molecules using four tumor cell lines: A549 (lung cancer), HCT116 (colorectal cancer), Namalwa (Burkitt lymphoma) and A375 (melanoma). Exponentially growing cells were treated with the compounds for 72 hours and inhibition of proliferation was evaluated with a conventional MTT assay. All hemi-synthetic compounds, except one, were found to be inactive (IC_{50} > 10 µM).

The only active molecule is compound 22 that proved to be roughly as potent as ANS. This compound is a robust cytotoxic agent, with an IC_{50} in the range 44-87 nM. This suggests that the shift of the acetyl group on the pyrrolidine-3,4-diol unit from one OH group to the other one does not alter the cytotoxic potential of the compound

Compound	**Protein Synth. Inh.**		**Cytotoxicity** (IC_{50}, nM)[b]		
	(EC_{50}, nM)[a]	A549	HCT116	Namalwa	A375
ANS	32	38.5	57.2	92	57.5
22	100	87.5	67	77.7	44.4

(a) Protein synthesis was evaluated using a DLD-1 4Ub-Luc colon cancer cell line.
(b) Cytotoxicity measurements, after 72 hours of drug treatment, performed with 4 tumor cell lines.

Table 2: Protein synthesis inhibition and cytotoxicity of ANS and compound 22.

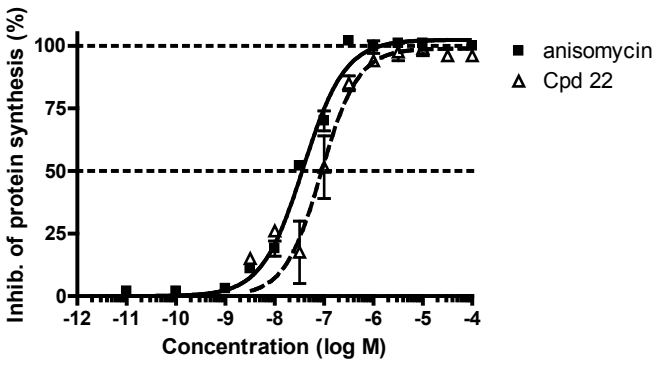


Figure 1: Dose-dependent inhibition of protein synthesis induced by ANS (filled square) and compound 22 (open triangles) in the DLD-1 4Ub-Luc colon cancer cell line.

whereas all the other structural modifications lead to a complete loss of activity. This compound 22 maintains the same cytotoxic effects of ANS. These observations prompted us to investigate further its activity with complementary cell assays. We analyzed the level of expression of proteins c-Myc, and cyclin-D1 in the breast cancer cell line MDA-MB-231 after a four hour treatment with ANS, or 22 at 0.1, and 1 µM (Figure 2).

The two compounds, ANS and 22 were found to be equally potent, both of them induce a dose-related inhibition of expression of the cell cycle regulator cyclin-D1 and the upstream regulator c-Myc. In particular, the effect on cyclin-D1 was very high, with a complete inhibition of protein expression at 1 µM.

Compound 22 was also found to decrease the migration and invasion of MDA-MB-231 cells. This effect was characterized using Boyden chamber and wound healing assays (Figure 3).

The anti-invasive activity of compound 22 is slightly less than that of ANS but highly significant. It is worth mentioning that this effect was observed at non-cytotoxic doses of 22 (after a 24 hour treatment), suggesting that inhibition of the cancer cell migration is not due to a cytotoxic effect. The same data were obtained with another cell line, SNB-78 glioblastoma.

Conclusions

ANS interferes with *de novo* protein synthesis via translational inhibition and as such, it is frequently used as a ribotoxic stress inducer. Its cytotoxic activity is associated with a marked inhibition of global protein synthesis, as shown here. To delineate further structure-activity relationships around this natural product, we have performed the hemi-synthesis of 18 derivatives and evaluated their effects on different cancer cell lines, in terms of cell proliferation, migration or invasion. Our study has led to the identification of one ANS derivative, compound 22 that acts as a potent inhibitor of protein synthesis and that reduces the expression of proteins cyclin-D1 and c-Myc with an efficacy close to that of the natural product. This compound 22 is also endowed with anti-migration and anti-invasion properties. All the other derivatives are much less active or inactive, indicating that an optimization of the pyrrolidine scaffold by modification of three substituents (R_1, R_2 and R_3) is a challenge. Previous SAR studies in this series have also led to similar conclusions. For example, it was shown that the biological activities of ANS derivatives dramatically dropped when the methoxy substituent was only moved to the *meta* or *ortho* positions to the benzyl group [24]. Similarly, another SAR study indicated that ANS inhibited protein synthesis by 75% at 1 µM but none of the derivatives inhibited protein synthesis.[25] Unsurprisingly, Nature has optimized the structure of the pyrrolidine alkaloid making it a potent immuno-suppressive agent with no significant side effects at effective therapeutic doses [15]. Compound **22** is a close analogue of ANS with an inversion of the OH and OAc groups at the 3, 4 positions, with the same stereo-configuration. This study provides useful information to better understand the SAR of ANS, and related pyrrolidine alkaloids (e.g. preussin).

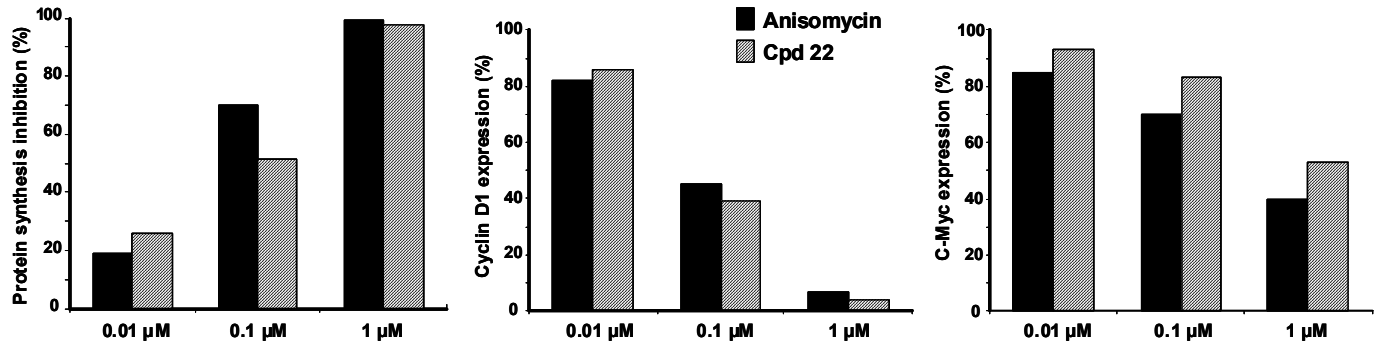


Figure 2: Comparison of the effects of ANS and compound 22 on protein synthesis in DLD-1Luc-4Ub cells and effects on the expression of c-Myc and cyclin-D1 in MDA-MB-231 cells. Experiments were performed twice. For protein expression, after migration on SDS-Page, dots were quantified by pixel recording and results were expressed as percentage of control (solvent 100 %).

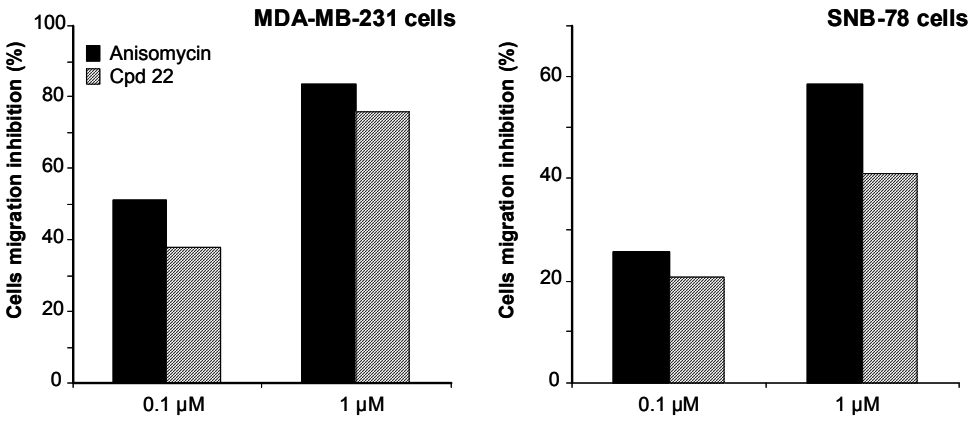


Figure 3: Comparison of the effects of ANS (filled bars) and compound 22 (hatched bars) on the migration of two cancer cell types: MDA-MB-231 breast cancer cells and SNB-78 glioblastoma cells.

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References

1. Lynch JE, English AR, Bauck H, Deligianis H (1954) Studies on the in vitro activity of anisomycin. Antibiot Chemother (Northfield) 4: 844-848.
2. Beereboom JJ, Butler K, Pennington FC, Solomons IA (1965) Anisomycin. I. determination of the structure and stereochemistry of anisomycin. J Org Chem 30: 2334-2342.
3. Xing F, Yu Z, Liu J, Di J, Zeng S, et al. (2008) Anisomycin inhibits the behaviors of T cells and the allogeneic skin transplantation in mice. J Immunother 31: 858-870.
4. Okulski P, Hess G, Kaczmarek L (2002) Anisomycin treatment paradigm affects duration of long-term potentiation in slices of the amygdala. Neuroscience 114: 1-5.
5. Torocsik B, Szeberenyi J (2000) Anisomycin uses multiple mechanisms to stimulate mitogen-activated protein kinases and gene expression and to inhibit neuronal differentiation in PC12 phaeochromocytoma cells. Eur J Neurosci 12: 527-532.
6. Torocsik B, Szeberenyi J (2000) Anisomycin affects both pro- and antiapoptotic mechanisms in PC12 cells. Biochem Biophys Res Commun 278: 550-556.
7. Jin CY, Park C, Hong SH, Han MH, Jeong JW, et al. (2013) Synergistic induction of TRAIL-mediated apoptosis by anisomycin in human hepatoma cells via the BH3-only protein Bid and c-Jun/AP-1 signaling pathway. Biomed Pharmacother 67: 321-328.
8. You P, Xing F, Huo J, Wang B, Di J, et al. (2013) In vitro and in vivo evaluation of anisomycin against Ehrlich ascites carcinoma. Oncol Rep 29: 2227-2236.
9. Seo BR, Min KJ, Kim S, Park JW, Park WK, et al. (2013) Anisomycin treatment enhances TRAIL-mediated apoptosis in renal carcinoma cells through the down-regulation of Bcl-2, c-FLIP(L) and Mcl-1. Biochimie 95: 858-865.
10. Slipicevic A, Øy GF, Rosnes AK, Stakkestad Ø, Emilsen E, et al. (2013) Low-dose anisomycin sensitizes melanoma cells to TRAIL induced apoptosis. Cancer Biol Ther 14: 146-154.
11. Liu Y, Ge J, Li Q, Gu L, Guo X, et al. (2013) Anisomycin induces apoptosis of glucocorticoid resistant acute lymphoblastic leukemia CEM-C1 cells via activation of mitogen-activated protein kinases p38 and JNK. Neoplasma, 60: 101-110.
12. Li JY, Huang JY, Li M, Zhang H, Xing B, et al. (2012) Anisomycin induces glioma cell death via down-regulation of PP2A catalytic subunit in vitro. Acta Pharmacol Sin 33: 935-940.
13. Mawji IA, Simpson CD, Gronda M, Williams MA, Hurren R, et al. (2007) A chemical screen identifies anisomycin as an anoikis sensitizer that functions by decreasing FLIP protein synthesis. Cancer Res 67: 8307-8315.
14. Monaghan D, O'Connell E, Cruickshank FL, O'Sullivan B, Giles FJ, et al. (2014) Inhibition of protein synthesis and JNK activation are not required for cell death induced by anisomycin and anisomycin analogues. Biochem Biophys Res Commun 443: 761-767.
15. Tang Z, Xing F, Chen D, Yu Y, Yu C, et al. (2012) In vivo toxicological evaluation of Anisomycin. Toxicol Lett 208: 1-11.
16. Hulme AN, Rosser EM (2002) An aldol-based approach to the synthesis of the antibiotic anisomycin. Org Lett 4: 265-267.
17. Rosser EM, Morton S, Ashton KS, Cohen P, Hulme AN (2004) Synthetic anisomycin analogues activating the JNK/SAPK1 and p38/SAPK2 pathways. Org Biomol Chem 2: 142-149.
18. Kim JH, Curtis-Long MJ, Seo WD, Ryu YB, Yang MS, et al. (2005) Stereodivergent syntheses of anisomycin derivatives from D-tyrosine. J Org Chem 70: 4082-4087.
19. Shi S, Zhu S, Gerritz SW, Esposito K, Padmanabha R, et al. (2005) Solid-phase synthesis and anti-infective activity of a combinatorial library based on the natural product anisomycin. Bioorg Med Chem Lett 15: 4151-4154.
20. Inverarity IA, Viguier RF, Cohen P, Hulme AN (2007) Biotinylated anisomycin: a comparison of classical and "click" chemistry approaches. Bioconjug Chem 18: 1593-1603.
21. Joo JE, Lee KY, Pham VT, Tian YS, Ham WH (2007) Application of Pd(0)-catalyzed intramolecular oxazine formation to the efficient total synthesis of (-)-anisomycin. Org Lett 9: 3627-3630.
22. Goard M, Aakalu G, Fedoryak OD, Quinonez C, St Julien J, et al. (2005) Light-mediated inhibition of protein synthesis. Chem Biol 12: 685-693.
23. Vandenberghe I, Créancier L, Vispé S, Annereau JP, Barret JM, et al. (2008) Physalin B, a novel inhibitor of the ubiquitin-proteasome pathway, triggers NOXA-associated apoptosis. Biochem. Pharmacol., 76: 453-462.
24. Hall SS, Loebenberg D, Schumacher DP (1983) Structure-activity relationships of synthetic antibiotic analogues of anisomycin. J Med Chem 26: 469-475.
25. Jacklet JW (1980) Protein synthesis requirement of the Aplysia circadian clock. Tested by active and inactive derivatives of the inhibitor anisomycin. J Exp Biol 85: 33-42.
26. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, et al. (2013) In vitro cell migration and invasion assays. Mutat Res 752: 10-24.