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SEMI-SYNTHETIC MITHRAMYCIN DERIVATIVES WITH ANTI-CANCER ACTIVITY

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/737,353 filed December 14, 2012, the entire disclosure of which is hereby incorporated by reference herein.

TECHNICAL FIELD

[0002] The present disclosure relates to mithramycin side chain carboxylic acid (MTM SA) derivatives and their use in the treatment of cancers.

BACKGROUND

[0003] Natural products provide some of the most promising and effective known anti-cancer agents. Even if not used directly, they offer a template to derivatize and/or mimic. Natural products and their derivatives make up more than 60% of all clinically used anti-cancer agents. However, natural products are often not optimized for human diseases and can in turn interact with many normal cells causing unwanted side effects. Biosynthetic and chemical modifications of natural products offer a great opportunity to improve the anti-cancer properties of natural product drugs while reducing their non-specific interactions.

[0004] For example, mithramycin (MTM) is an aureolic acid type anti-cancer agent produced by various soil bacteria of the genus *Streptomyces*. MTM exhibits compelling anticancer activity and a unique mode of action. MTM has a clinical history, e.g., it has been investigated in applications for the treatment testicular cancer, Paget bone disease, and hypercalcemia, but has been limited by side effects such as hepatic, gastrointestinal, bone marrow, and renal toxicities. MTM has shown promise with respect to treating neurological diseases, glioblastomas, and other tumors in addition to showing the ability to inhibit the multi-drug resistance efflux pump MDR1 for which smaller, less toxic doses are required. Most recently MTM was identified as the lead compound against the Friend leukemia virus integration 1(EWS-FLI1) transcription factor and in a combinational approach with betulinic acid to treat pancreatic cancer.

[0005] It is clear that MTM has high potential in the fight against cancer and new and improved analogues would find clinical relevance. A need thus exists to improve the performance and efficacy of MTM.

SUMMARY OF THE DISCLOSURE

[0006] An advantage of the present invention is MTM SA derivatives or their pharmaceutically acceptable salts. Such derivatives can be used in the treatment of various cancers and various neuro-diseases.

[0007] Additional advantages of the present invention will become readily apparent to those skilled in this art from the following detailed description, wherein only the preferred embodiment of the invention is shown and described, simply by way of illustration of the best mode contemplated of carrying out the invention. As will be realized, the invention is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the invention. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Reference is made to the attached drawings, wherein elements having the same reference numeral designations represent similar elements throughout and wherein:

[0009] Fig. 1 is a schematic representation of the accumulation of MTM SK, MTM SDK, and MTM SA by the inactivation of the *mtmW* gene in the MTM biosynthetic pathway.

[0010] Fig. 2 illustrates two ways to produce the starting material MTM SA and also shows various MTM SA derivatives.

[0011] Fig. 3 illustrated functionalization of MTM SA through a reaction with a primary amine containing compound. Representative compounds are shown.

[0012] Fig. 4 is a chart illustrating the in vitro cytotoxicity assays of the three most active MTM SA derivatives.

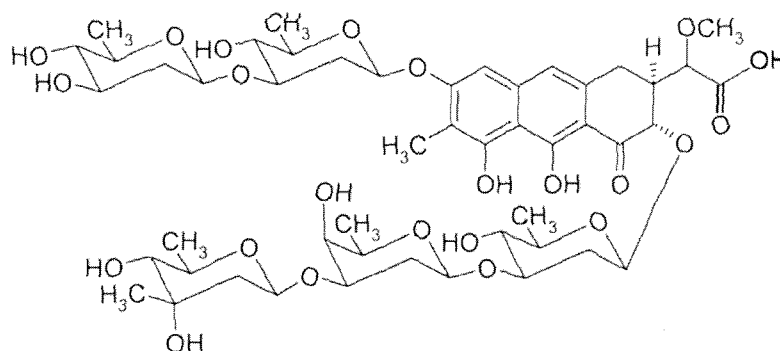
DETAILED DESCRIPTION OF THE DISCLOSURE

[0013] MTM is an example of how modifying a natural product can yield improved anticancer agents. It is believed that MTM acts by cross-linking GC-rich DNA thereby shutting down the transcription of several proto-oncogenes, particularly pathways regulated by transcription factors Sp1 and Sp3. The Sp1 transcription factors are important as they have been linked to the control of cell growth, survival, and differentiation and their overexpression has been observed in several cancers.

[0014] Extensive combinatorial biosynthesis has been performed on the drug biosynthesis pathway to produce altered MTM analogues for the purpose of improving their toxicity profiles. This has resulted in several novel useful compounds.

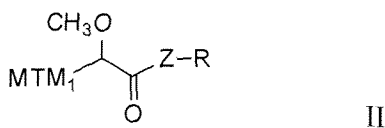
[0015] The inactivation of the *mtmW* gene, which is the gene encoding the last acting enzyme in the MTM biosynthetic pathway, produced the analogues MTM with a short side chain ketone (SK) and MTM with a short side chain diketone (SDK) (Figure 1). Both of these analogues possess shorter side chains at the 3-position. The 3-side chain has been identified previously as important, since it is in part responsible for MTM's interaction with the DNA phosphate backbone. See U.S. Patent No. 7,423,008. Both MTM SK and MTM SDK showed increased activity against several cancer cell lines compared to the parent MTM. These results indicate that the 3- side chain is important for the activity of MTM and offers a base for further molecular manipulations. As an unwanted side product along with the production of the desired MTM SK and MTM SDK analogues, MTM side chain carboxylic acid (SA) is also accumulated in the *MtmW*-minus-mutant, but showed in contrast to MTM SK and MTM SDK significantly decreased activity compared to MTM.

[0016] MTA SA is shown in formula (I) below:

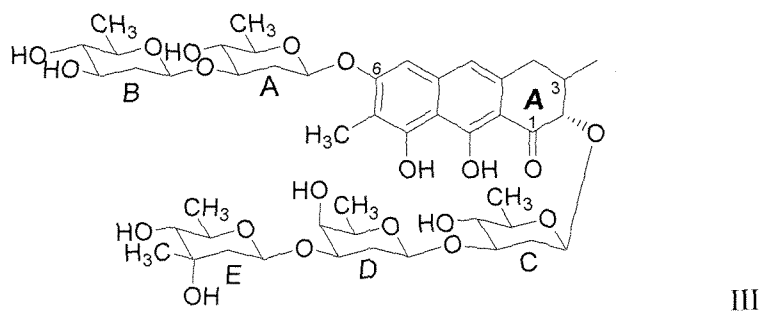


(I)

[0017] One reason for MTM- SA's decreased activity might be that its 3-side chain is too short and its negatively charged carboxylic acid does not sufficiently interact with naturally negatively charged DNA. To overcome these potential deficiencies, we used a semi-synthetic approach to chemically modify the unique carboxylic acid moiety of MTM SA to introduce new functionalities into the 3-side chain. In one aspect of the present disclosure, the MTM SA derivatives have the following formula:



[0018] where Z represents O, S, N-R'; R and R' represent, for each occurrence, H, alkyl, e.g., lower straight chain or branched alkyl, heterocyclic, aryl, e.g., phenyl, naphthyl, heteroaryl, e.g., pyridyl, pyrrolidyl, piperidyl, pyrimidyl, indolyl, thienyl, provided that R is not H when Z is O; ZR taken together represents an organic residue, e.g., an alkyl, e.g., lower straight chain or branched alkyl, heterocyclic, aryl, e.g., phenyl, naphthyl, heteroaryl, e.g., pyridyl, pyrrolidyl, piperidyl, pyrimidyl, indolyl, thienyl, an amino acid conjugate or its ester derivative, e.g., proline (Pro), alanine (Ala), serine (Ser), cysteine (Cys), histidine (His), tryptophan (Trp), tyrosine (Tyr), conjugate, etc., a sugar or sugar chain. Each of the alkyl, heterocyclic, aryl, heteroaryl, sugar, or sugar chain of R, R' and ZR can be unsubstituted or substituted with one or more amino, alkyl amino, alkylcarboxyl, alkoxy, alkylcarbonyl, hydroxyl, hydroxyl, thio, alkyl disulfide, heterocyclic, aryl, heteroaryl, halo, e.g., fluoro, chloro, bromo, iodo, an amino acid conjugate, ether, ester, amide residue, etc. The group MTM₁ represents the fused ring portion of the mithramycin structure and can include different sugars or sugar chains. In other words, MTM₁ represents the structure of formula III below, but also variants with different sugar patterns.

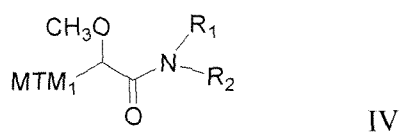


[0019] Thus, the A, B, C, D, E, sugars can be different from those shown, and include chain variants. Such sugars are disclosed, for example, in: (a) Baig, I.; Pérez, M.; Braña, A. F.; Gomathinayagam, R.; Damodaran, C.; Salas, J. A.; Méndez, C.; Rohr, J., Mithramycin analogues generated by combinatorial biosynthesis show improved bioactivity. *J. Nat. Prod.* **2008**, 71 (2), 199-207; (b) Pérez, M.; Baig, I.; Braña, A. F.; Salas, J. A.; Rohr, J.; Méndez, C., Generation of new derivatives of the antitumor antibiotic mithramycin by altering the glycosylation pattern through combinatorial biosynthesis. *ChemBioChem* **2008**, 9 (14), 2295-

2304; (c) Nuñez, L. E.; Nybo, S. E.; Gonzalez-Sabin, J.; Pérez, M.; Ménendez, N.; Braña, A. F.; He, M.; Morís, F.; Salas, J. A.; Rohr, J.; Méndez, C., A Novel Mithramycin Analogue with High Antitumor Activity and Less Toxicity Generated by Combinatorial Biosynthesis. *J. Med. Chem.* **2012**, 55, 5813-5825; (d) Remsing, L. L.; Garcia-Bernardo, J.; Gonzalez, A. M.; Künzel, E.; Rix, U.; Braña, A. F.; Bearden, D. W.; Méndez, C.; Salas, J. A.; Rohr, J., Ketopremithramycins and ketomithramycins, four new aureolic acid-type compounds obtained upon inactivation of two genes involved in the biosynthesis of the deoxysugar moieties of the antitumor drug mithramycin by *Streptomyces argillaceus*, reveal novel insights into post-PKS tailoring steps of the mithramycin biosynthetic pathway. *J. Am. Chem. Soc.* **2002**, 124 (8), 1606-1614; (e) Remsing, L. L.; Bahadori, H. R.; Carbone, G. M.; McGuffie, E. M.; Catapano, C. V.; Rohr, J., Inhibition of c-src transcription by mithramycin: structure-activity relationships of biosynthetically produced mithramycin analogues using the c-src promoter as target. *Biochemistry* **2003**, 42 (27), 8313-8324. Pharmaceutically acceptable salts of the MTM SA derivative are also contemplated by the present disclosure.

[0020] Preferably, Z represents NH, O, or S and R represents alkyl, aryl, heterocyclic, heteroaryl, etc., and when ZR is taken together, ZR represents an amino acid conjugate or its ester (e.g., a methyl ester).

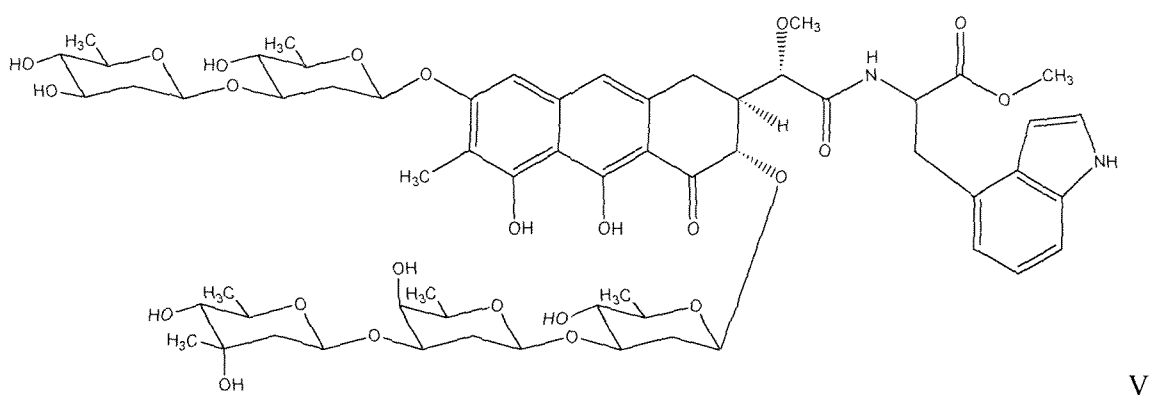
[0021] Previous work by Preobrazhenskaya et al. (References numbered 26-28) on olivomycin derivatizations showed that introduction of N-atoms can improve aureolic acid type anticancer drugs. We discovered that MTM SA derivatives with N-atoms can have improved efficacy. In one embodiment of the present disclosure, MTM SA is derivatized with an amine to form an amide. Such MTM SA amide derivatives will advantageously have an elongated 3-side chain and one or more N-atom/s, which can enhance the interaction of the derivative with the DNA-phosphate backbone resulting in improved efficacy. In one aspect of the present disclosure, the MTM SA derivative is provided by formula (IV) below:



where R₁ and R₂ can be the same or different and each of R₁ and R₂ can be an H, an amino acid conjugate, e.g., proline (Pro, or O-alkyl-Pro), alanine (Ala, or O-alkyl-Ala), serine (Ser, or O-alkyl-Ser), cysteine (Cys, or O-alkyl-Cys), histidine (His, or O-alkyl-His), tryptophan (Trp, or O-alkyl-Trp), tyrosine (Tyr, or O-alkyl-Tyr), conjugate, a lower straight chain or branched alkyl

unsubstituted or substituted with one or more amino, alkyl amino, alkylcarboxyl, alkoxy, alkylcarbonyl, hydroxyl, thio, alkyldisulfide, halo, e.g., fluoro, chloro, bromo, iodo, provided that R_1 and R_2 are not both H simultaneously.

[0022] In one embodiment of the present disclosure, the MTM SA derivative is an amino acid derivative, i.e., at least one of R_1 or R_2 is an amino acid conjugate, e.g., proline (Pro), alanine (Ala), serine (Ser), cysteine (Cys), histidine (His), tryptophan (Trp), tyrosine (Tyr), conjugate. Preferably R_1 is an amino acid conjugate and R_2 is H. For example, the MTM SA derivative can be a MTM SA O-Me-tryptophan derivative as shown in formula V below:



[0023] Figure 2 illustrates two different ways to produce the starting material MTM SA and also shows various MTM SA derivatives. In the figure, the MTM_i group can include different sugars or sugar chains, R^2 represents short amide or amino acid derived side chains etc., in which R^3 can be H, CH_3 , C_2H_5 , Ac ($= COCH_3$), or other ether or ester or amide residues. In one aspect of the present disclosure, MTM SA amide derivatives can be prepared by coupling the terminal carboxylic acid group of MTM SA with an amine, e.g., a primary amine, to form the MTM SA derivative.

[0024] Examples of particular MTM SA derivatives together with their activity are provided in Table 2 in the Examples section below. Additionally, other amino acid derivatives of MTM SA can be prepared. For example, tryptophan can be substituted on the MTM SA derivative. The MTM SA-tryptophan derivative can be prepared the same way as the other amino acid derivatives, only using Tryptophan-O-methyl ester. Also OMe-Phe, OMe-Tyr and OMe-His derivatives can be prepared in the same manner.

[0025] The MTM SA derivatives of the present disclosure can be used for the treatment of cancer, such as brain, colon, prostate, lung, breast, esophageal, pancreatic, skin, Ewing sarcoma, any type of blood cancer etc. MTM derivatives are also neuroprotective and the MTM SA derivative can be used to treat various neuro-diseases, such as Huntington disease, etc.

[0026] In another aspect of the present disclosure, an effective amount of the MTM SA derivative or a pharmaceutically acceptable salt thereof is administered to a patient in need of cancer treatment or a neuro-disease. The MTM SA derivatives or pharmaceutically acceptable salts thereof of the present disclosure can be administered to a patient, e.g., a human patient, in need of such treatment by any route. The MTM SA derivatives or pharmaceutically acceptable salts thereof of the present disclosure can be administered alone or with a pharmaceutically acceptable carrier or excipient.

[0027] The biosynthesis of MTM SK and MTM SDK is accomplished through a genetically engineered *S. argillaceus* strain, M7W1, which contains an inactivated *mtmW* gene coding for the MtmW enzyme. Both the MTM SK and MTM SDK analogues have improved activity compared to the parent MTM compound, thus it would be optimal if these were the only two compounds produced by the M7W1 strain. However, this is not the case, and two other major compounds are produced alongside of MTM SK and MTM SDK. One of these compounds, MTM SA, has previously been disregarded as invaluable due to the relative lack of biological activity compared to the parent compound. This is unfortunate as MTM SA is produced in many fermentations in higher amounts than MTM SK or MTM SDK, and the production yield can be shifted even further in favor of the production of MTM SA by altering the pH of the culture media. Since MTM SK and MTM SDK are separated chromatographically MTM SA is easily collected and isolated alongside MTM SK and MTM SDK during the normal isolation procedure.

[0028] An aspect of the present disclosure involves targeting the 3-side chain of MTM SA to form useful MTM SA derivatives. It is known that the 3-side chain of the MTM structure is responsible for an interaction with the DNA-phosphate backbone. Thus by altering the functionality of the 3-side chain the specificity for the DNA of diseased cells can be improved. The 3-side chain of MTM SA is terminated by a carboxyl acid functional group which is likely ionized at a physiological pH, repulsing from the negative charge of the DNA phosphate backbone thereby weakening MTM SA's ability to bind to the DNA.

[0029] In one aspect of the present disclosure, side chain functionalizations were rationally selected to contain cationic amine residues in order to enhance the interaction with DNA phosphate backbone. Both MTM SK and MTM SDK have shorter side chains than MTM. It was unclear whether modifications of adding an N-atom and also chain length would have an overall positive effect. To test this several different side chain modifications were investigated with differing lengths and additional functionalities (Figure 3).

[0030] Various reaction conditions were examined before discovering an optimal protocol. The mass, UV absorptions and retention times of the products separated by HPLC were used to identify the products. Initial reactions were performed in DMF with the different coupling agents. These reactions tended to result in multiple product peaks. The peaks typically had the expected mass or the expected mass plus one additional side chain. MTM, MTM SK, and MTM SDK have a chromophore which absorbs light at roughly 410 nm. The UV-absorption of the different products was typically either 410 nm as expected, or in the 450 nm range, which was not expected. The products that had a mass corresponding to two side chains being attached also showed the 450 nm absorption. This led us to believe that a second side chain was being attached somewhere on or in the proximity of the chromophore, although it was not clear as to exactly where or how this was happening. It could be possible that DMF and excess side chain promoted conditions capable of allowing the carbonyl group of the A-ring to be attacked by the amine side chain, however this was not confirmed. These reactions also typically had one product that did match the expected mass and UV absorption profile although it was not always the major product. Once DCM was substituted as the reaction solvent the number of reaction products was typically reduced to one with the correct mass and UV. The retention times also matched with the product from the DMF reaction also having the correct mass and UV absorption. PyBOP outperformed the other couple agents (COMU, DPPA, and TBTU) in terms of productions yield and reaction time. With COMU, DPPA, and TBTU there was unreacted MTM SA left in the product mixture even with excess side chain molecules and coupling reagents. However when PyBOP was used in combination with DCM only minute traces of MTM SA was observed in the production chromatograph. Early reactions were also allowed to proceed for up to 48 h before stopping the reaction, still with unreacted MTM SA.

[0031] However, when PyBOP was used reactions were complete within 12 h. All initial reactions were done on a small scale of only 2 mg MTM SA in order to optimize the reaction conditions without wasting precious reagents. For these reasons DCM and PyBOP were chosen as the optimal solvent and coupling reagent for the subsequent reactions and scaled up production.

[0032] To test the effectiveness of the side chain modifications, cytotoxicity assays were performed against the human non-small cell lung cancer cell line (A549). The least effective side chain modification was with cystamine as it eliminated all cytotoxicity effects against the cell line. The modification of the side chain with methyl hydrazine did not improve the cytotoxicity of the molecule compared to MTM SA, but it should be noted that it also did not

decrease the activity either. A modest 3-4 fold increase in activity was observed with the N,N-dimethylethylenediamine functionalization. The increase in activity was still well below the value of MTM or MTM SK. The modification of the side chain with O-methyl protected amino acids residues yielded much better results. L-Ala showed the greatest activity with >23-fold improvement over MTM SA and an IC₅₀ value very near to that of MTM SK. L-Gly and L-Val also showed good results with a 15- and 10-fold improvement, respectively. L-Cys did also show an 8-fold improvement over MTM SA.

[0033] The cytotoxicity improvement with L-Cys is not as good as some of the other analogues, however this and similar modifications have the potential to be of value for a different reason. Producing a potent anticancer molecule is only half of the battle in the fight of cancer. The other half is being to get the drug to the area of interest in high enough concentrations to be effective without damaging the rest of the body and healthy tissues. We have reported previously the development of a nanoparticle delivery system compatible with MTM SK and MTM SDK to complement the potency of these molecules and enhance the cytotoxicity. The unique thiol group introduced with the L-Cys side chain offers the potential to load this molecule through a disulfide bond into a compatible delivery system. This bond would be stable throughout the blood stream with low glutathione concentrations, however once the delivery system was internalized by the cancer cells and the system was exposed to the increased intracellular glutathione concentrations (up to the millimolar concentration) which would reduce the disulfide bond and release the L-Cys functionalized MTM analogue. Several drug delivery systems have previously shown the ability to take advantage of the increased intracellular glutathione concentration to modulate drug release. The fact that less drug would be circulating systemically and interacting with normal tissue may allow a higher dose of drug to be administered and overcome the lower cytotoxicity of the molecule compared to MTM SK. The L-Cys modified MTM SA is still a very active compound compared to other anti-cancer agents.

[0034] While working on the modifications of MTM SA, Preobrazhenskaya et al. published the side chain derivatives of another aureolic acid antibiotic, olivomycin A. Here the side chain of olivomycin A was chemically shortened, to achieve olivomycin SA, and additionally functionalized as amides with selected amines. The authors observed favorable results when the modifications of the side chain were achieved using N,N-dimethylethylenediamine and O- methyl alanine. As described above, the latter modification also improved MTM SA. However, the reported methods are not compatible with MTM, because an oxidative sodium periodate cleavage was used to cut the side chain to olivomycin

SA. Applying that periodate cleavage to MTM would also destroy the two terminal sugar residues (sugars B and E), thereby interfering with two structural elements important for the MTM bioactivity. We circumvented the problem by using biotechnologically produced MTM SA followed by synthetic modifications similar to those reported by Preobrazhenskaya et al.

EXAMPLES

[0035] The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein.

[0036] Materials and Methods

[0037] N,N'-diisopropylcarbodiimide (DIC), N-hydroxysuccinimide (NHS), dimethylformamide (DMF), dichloromethane (DCM), O-(Benzotriazol-1-yl)-N, N, N', N''-tetramethyluronium tetrafluoroborate (TBTU), COMU, diphenyl phosphoryl azide (DPPA), N,N-diisopropylethylamine (DIPEA), methyl hydrazine, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), L-cysteine methyl ester hydrochloride, L-glycine methyl ester hydrochloride, L-alanine methyl ester hydrochloride, L-valine methyl ester hydrochloride, cystamine, DMSO (molecular biology grade, 99.9 %), dimethylsulfoxide-d₆ (DMSO-d₆), resazurin sodium salt, were purchased from Sigma-Aldrich (USA). N,N-dimethylethylenediamine was purchased from TCI America (USA). 1- amino-2-propanone was purchased from Waterstone Tech (USA). Methanol (MeOH), acetonitrile (ACN), celite, C18 RP silica gel, tryptic soy broth (TSB), LB broth, Difco agar, sucrose, potassium sulfate, magnesium chloride, glucose, casamino acids, yeast extract, MOPS, and trace elements were purchased from Fisher Scientific (USA). *Streptomyces argillaceus* ATCC 12956, A549 tissue culture cells, F-12K media, fetal bovine serum (FBS) were purchased from ATCC (USA).

[0038] Biosynthesis of MTM SA

[0039] MTM SA was produced by an adapted procedure reported previously. *S. argillaceus* M7W1 was plated on R5A agar and allowed to grow for four days or until spores formed. The spores were then used to seed a culture in 100 mL of tryptic soy broth (TSB) and grown for 24 hr in an orbital shaker at 28 °C, 250 rpm. After 24 hrs 4 mL of the TSB culture was used to start a culture in R5A media in 40, 100 mL flasks. The cultures in R5A media were grown for 3 days at 28 °C, 250 rpm while the production of SA was monitored by HPLC. After 3 days the cells were collected with 50 g/L of celite and removed by filtration. The cell

pellet was then re-dissolved in MeOH and sonicated for 1 hr to lyse the cells. Following the cell lysis the cellular debris was filtered off and the MeOH was evaporated from the filtrate. The dried cellular extract was then reconstituted in water and loaded onto a 5 x 12 cm C18 RP column equilibrated with 10 column volumes of water. The column was washed with 10% ACN in water, followed by a fractionation of ACN in water from 20-50%, followed by 100% ACN. The filtrate removed from the cells in step one was added to the C18 column and eluted by the same fractionation procedure. The samples were dried and re-constituted in 80% MeOH and water. SA was then completely isolated from the mixture of a few compounds by semi-preparative HPLC.

[0040] **Synthetic Modification of MTM SA 3 Side Chain**

[0041] MTM SA was modified by converting the terminal carboxylic acid group with a primary amine through a coupling reaction. Several different protocols were investigated to discover the optimal reaction conditions. For all of the exploratory reactions 2 mg of MTM SA was used. The initial reaction was completed by reacting MTM SA with the desired side chain modifying molecule in a 3x molar ratio, 3x DIPEA and 1x TBTU in 500 DCM starting at 4 °C. The reaction was monitored at 2 h by HPLC-MS and allowed to proceed a total 24 h with the products analyzed by HPLC-MS. For HPLC-MS analysis a small aliquot was removed from the reaction mixture, the solvent dried off, and then reconstituted in methanol for analysis. For the initial reaction an extract on with ethyl acetate and the separation of the aqueous and organic phase was attempted, however this was not repeated with the subsequent reactions. The reaction was then repeated with 3x DIPEA, 10x of the side chain modification molecule, and 2x of the coupling agent DPPA in DMF, starting at 4 °C and monitored at 24 and 36 h. The protocol was modified and optimized by returning to 3x of the desired side chain modification molecule and while substituting the coupling agents COMU or PyBOP and the solvent DCM or DMF in different combinations. The reactions were checked at 24 and 48 h. After the reactions were completed the solvent was removed and the product mixture was reconstituted in methanol. The mixture was analyzed by HPLC-MS using a combination of the mass and UV absorbance to identify the elution peaks corresponding to the expected products. HPLC was then used to isolate the individual compounds. The organic solvents were removed from the samples followed by the freeze drying of the compounds. To scale up the production of the compounds 10 mg of SA was reacted with 3x of the side chain molecule, 3x DIPEA, 2x PyBOP in DCM, starting at 4 °C, for 24 h. The solvent was removed and the products were reconstituted in methanol and isolated by HPLC. The side chain modification molecules included methyl hydrazine, L-cysteine methyl

ester, L-glycine methyl ester, L-alanine methyl ester, L-valine methyl ester, cystamine, N,N-dimethylethylenediamine, and 1-amino-2-propanone (Figure 2).

[0042] **Structure Confirmation**

[0043] The structures of the most active derivatives from the reactions with MTM SA and L-glycine methyl ester, L-alanine methyl ester, and L-valine methyl ester were confirmed through ^1H and ^{13}C NMR along with MALDI-TOF mass spectrometry performed by the University of Kentucky Mass Spectrometry Facility. The NMR measurements were taken on a 500 MHz NMR in methanol- d_4

[0044] **In Vitro Cytotoxicity Assays**

[0045] The cytotoxic effects of the side chain modifications of SA were investigated in order to determine whether the modification were beneficial to the drug structure. All cytotoxicity assays were performed with A549, human non-small cell lung cancer cells. A549 cells were cultured as specified from ATCC at 37 °C, 5% CO_2 . The cells were added to a 96 well plate (5,000 cells/well) and permitted to attach for 24 h. After 24 h culture media were replaced with the side chain modified MTM SA derivative containing media at differing concentrations. The cells were incubated with the drug containing media for 72 hrs total (n=8). Cell viability was determined using a resazurin assay that signifies mitochondrial metabolic activity in living cells. 10 μL of a 1 mM resazurin solution in PBS was added to the control and analogue-treated cells at the end of the treatment period. Cell viability was determined three hours later by reading the fluorescence at 560 nm (Ex)/ 590 nm (Em). The fluorescence signals were quantified using a Spectramax M5 plate reader (Molecular Devices) equipped with a SoftMaxPro software. Cytotoxicity was determined by calculating the half maximal inhibitory concentration (IC_{50}) of each sample.

[0046] **RESULTS**

[0047] **Biotechnological production of MTM SA**

[0048] MTM SA was successfully produced by the *S. argillaceus* M7W1, and isolated through an adapted procedure developed previously. Spores of *S. argillaceus* were formed by plating the cells of the M7W1 mutant strain on R5A agar and allowing them to grow until spores were formed. Colonies of the *S. argillaceus* cells began to appear after incubation for two days and after three days spores were observed. The cells were allowed to incubate for one more day to allow the majority of the cells to exist as spores and then added to TSB media. Once transferred to the TSB the cells grew quickly and after 24 h the TSB culture was used to inoculate multiple flasks of R5A media to produce SA. The pH of the R5A media was adjusted to pH 6.85. HPLC was used to monitor the culture for the production of SA. After 72 h

the culture production was primarily composed of the end products including MTM SK and MTM SDK in addition to MTM SA, and the culture was terminated. A large portion of the MTM SA production is excreted into the culture media so once the culture process was stopped the media and the cells were separated. Celite cell binding resin was used to bind the cells and allow the culture to more easily be separated through filtration. C18 RP silica gel was able to successfully collect MTM SA from the complex culture mixture. Other compounds were also collected by the column so initial washes of 10% and 20% ACN were used to *remove many of the byproducts* from the sample. Further fractionation of the culture on the C18 RP column in addition to the loading and subsequent fractionation on a new, smaller C18 RP column did not aid in the isolation of MTM SA from the other MTM analogues so all compounds were eluted together with 100% ACN to minimize dilution. There was also still some MTM SA contained inside the cells removed in the initial filtration step so the cells were lysed by sonication in methanol to release the intracellular content including all the MTM SA. Filtration removed the cellular debris, followed by the removal of the methanol. The same procedure as with the culture media then resulted in a relatively simple mixture of MTM SA along with the other MTM analogues and a few other impurities. Since the fractionation of the sample on the C18 RP silica gel did not completely isolate MTM SA from the other analogues the final purification steps were performed by using semi-preparative HPLC. The individual peak corresponding to MTM SA was collected and analyzed for purity using HPLC-MS. MTM SA was able to be collected at >95% purity and was stored at 20 °C until further use.

[0049] **Synthetic Modification of MTM SA 3 Side Chain**

[0050] Once produced MTM SA was modified chemically by taking advantage of the terminal carboxylic acid on the 3-side chain of the molecule. Several different protocols were attempted in order to discern the most favorable reaction conditions. The reactions were monitored by HPLC and the ratio of the signal corresponding to MTM SA was compared to the appearance of any new peaks following the reaction. MTM SA eluted with a retention time of 16.5 min, showing absorption of 410 nm and a mass to charge ratio of m/z 1026. The initial reaction with methyl hydrazine and TBTU as a coupling agent in DCM only displayed SA as the major component of the mixture after 2 h with only very minor other peaks appearing. After 24 h the MTM SA peak was gone with several broad peaks with shorter retention times appeared. The initial attempt to separate the products by ethyl acetate extraction did not result in favorable results and thus was not repeated. The reaction was then repeated with methyl hydrazide with the DPPA coupling agent in DMF. The reaction was also performed with 1-amino-2-propanone under the same conditions. After 24 h a new peak at 14.0

min appeared equal in intensity to that of the MTM SA peak. The expected product had a m/z of 1054.4, the same as the 14.0 min peak of 1054.4. The absorption of the peak was shifted however to 440 nm. The reaction with the 1-amino-2-propanone did not produce a new peak even after 36 hrs. The reaction was then repeated using the coupling agent COMU in DMF with the side chains L-glycine methyl ester hydrochloride and N,N-dimethylethylenediamine. For the reaction with N,N-dimethylethylenediamine a new product appeared with a retention time of 11.25 min, a UV-Vis absorption of 410 nm and a m/z matching that of the expected product of 1096.6 (1096.5 expected). For the reaction with L-glycine methyl ester hydrochloride three new products were formed with retention times of 14.4, 15.6, and 17.0 min. The UV-Vis absorption for the peaks were 455 nm, 455 nm, and 410 nm with 1097.3, 1168.4, and 1097.3 m/z , respectively. The calculated m/z for the correct product of the reaction with L-glycine methyl ester hydrochloride is 1097.4 m/z . There was still also a small peak corresponding to MTM SA. The reaction of MTM SA with cystamine was performed in DMF with COMU and resulted in the formation of a new peak at 9.0 min with absorption of 455 nm and m/z 1160.6. The theoretical m/z of the expected product was 1085.8 but if a disulfide bond were to form between an attached side chain molecule and a free side chain molecule the expected m/z would be 1160.4 m/z . The reaction solvent was then switched to DCM and L-alanine methyl ester hydrochloride was used as the side chain modification molecule. This reaction resulted in the formation of one new peak with a retention time of 18 min at roughly equal intensity to that of the MTM SA peak and a UV-Vis of 410 nm and a m/z of 1111.3. The calculated m/z of the expected molecule was 1111.4. The same conditions were used with L-valine methyl ester hydrochloride as well which resulted in a new molecule with a retention time of 20.6 min, a UV-Vis absorption of 410 nm and a m/z of 1139.5. The peak was the major product compared to the SA peak. The calculated m/z of the expected product was 1139.5. The reactions with L-glycine methyl ester hydrochloride and N,N-dimethylethylenediamine were then repeated with DCM as the solvent and PyBOP as the coupling agent. These reactions resulted in the formation of one new product with a retention time of 11.0 min for the N,N-dimethylethylenediamine reaction and 17.0 min for the L-glycine methyl ester hydrochloride reaction. Both products at a UV-Vis absorption of 410 nm and the m/z of the expected products identified previously. The individual peaks were collected from HPLC, the ACN removed, and freeze dried for use with the in vitro cytotoxicity assays.

[0051] **Structure Confirmation by NMR**

[0052] The structures of MTM SA-L-glycine methyl ester hydrochloride, MTM SA-L-alanine methyl ester hydrochloride, and MTM SA-L-valine methyl ester hydrochloride were

confirmed by H^1 and C^{13} NMR and are summarized in Table 1. The mass of the derivatives was also confirmed by mass spectrometry. For MTM SA-L-glycine methyl ester hydrochloride the expected mass + Na was 1120.45 and the observed mass was 1120.45, for MTM SA-L-alanine methyl ester hydrochloride the expected product had a calculated mass + Na of 1134.47 and the observed mass was 1134.47, and for MTM SA-L-valine methyl ester hydrochloride 1162.50 and observed mass was also 1162.50.

[0053] **In Vitro Cell Toxicity Assays**

[0054] The cytotoxicity of the MTM SA analogues was tested against the A549 non-small cell lung cancer cell line (Figure 4). Each analogue was checked individually with a range of concentrations to determine the IC_{50} of the molecule. For comparison sake the cytotoxicity of regular MTM, SA, and MTM SK, an analogue discovered previously and found to be more active than the regular MTM were also investigated.²² MTM SA showed an IC_{50} of 8.7 μ M, MTM SK an IC_{50} of 0.28 μ M, and MTM an IC_{50} of 0.18 μ M. The IC_{50} of the synthetically modified MTM SA analogues are as follows: MTM SA-methyl hydrazine: 8.8 μ M; MTM SA-N,N-dimethylethylenediamine: 2.7 μ M; MTM SA-cystamine: N/A; MTM SA-L-cysteine methyl ester hydrochloride: 1.0 μ M; MTM SA-L-glycine methyl ester hydrochloride: 0.55 μ M; MTM SA-L-alanine methyl ester hydrochloride: 0.36 μ M; MTM SA-L-valine methyl ester hydrochloride: 0.80 μ M (Table 2).

Table 2

The calculated IC_{50} values of the derivatives in vitro against the A549 and HT29 cell lines.

Side Chain Modifying Agent + SA	IC_{50} (μ M)	Cell Line
MTM	0.18	A549
MTM SK	0.28	A549
MTM SA	8.7	A549
Methyl hydrazine	8.8	A549
N,N-dimethylethylenediamine	2.7	A549
cystamine	N/A	A549
L-cysteine methyl ester	1.0	A549
L-glycine methyl ester	0.55	A549
L-alanine methyl ester	0.37	A549
L-valine methyl ester	0.80	A549
L-tryptophan methyl ester	0.27	HT29
L-serine methyl ester	1.72	HT29
MTM SK	0.43	HT29
MTM SDK	0.22	HT29

[0055] Natural products are not always optimized for human purposes. Combining biosynthetic derivatization with chemical synthesis produces unique molecules unattainable by either method individually. In this disclosure, we report the combination of these methods to modify the relatively inactive MTM SA that is accumulated alongside the biologically improved MTM analogues MTM SK and MTM SDK. The latter two molecules, which are both considerably more active and significantly less toxic than the natural product MTM itself, pointed us in the direction that 3-side chain modifications can be advantageous. The modification of the 3-side chain of MTM SA with amino acid derivatives yielded several active compounds with the O-methyl-alanine showing the most potent activity. The O-Me-alanine and the O-Me-glycine derivatives show activities comparable to MTM itself, and are clearly improved derivatives compared to the rather inactive MTM SA. Furthermore, this type of modification also allows the incorporation of important drug loading moieties for combination with specialized drug delivery systems.

[0056] Only the preferred embodiment of the present invention and examples of its versatility are shown and described in the present disclosure. It is to be understood that the present invention is capable of use in various other combinations and environments and is capable of changes or modifications within the scope of the inventive concept as expressed herein. Thus, for example, those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances, procedures and arrangements described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

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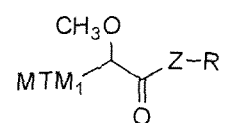
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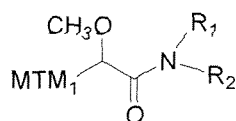
WHAT IS CLAIMED IS:

1. An MTM SA derivative or a pharmaceutically acceptable salt thereof.
2. The MTM SA derivative of claim 1 having the following formula:



or a pharmaceutically acceptable salt thereof; wherein Z represents O, S, N-R'; R and R' represent, for each occurrence, H, alkyl, heterocyclic, aryl, heteroaryl, an amino acid conjugate or its ester derivative provided that R is not H when Z is O; ZR taken together represents an organic residue; and wherein MTM₁ represents the fused ring portion of the mithramycin structure and can include different sugars or sugar chains.

3. The MTM SA derivative of claim 1 having the following formula:



wherein R₁ and R₂ can be the same or different and each of R₁ and R₂ can be an H, an amino acid conjugate, a lower straight chain or branched alkyl unsubstituted or substituted with one or more amino, alkyl amino, alkylcarboxyl, alkoxy, alkylcarbonyl, hydroxyl, thio, alkyl disulfide, halo, provided that R₁ and R₂ are not both H simultaneously.

4. The MTM SA derivative of claim 3, wherein the MTM SA derivative is an amino acid derivative.
5. The MTM SA derivative of claim 3, wherein R₁ is an amino acid conjugate and R₂ is H.
6. The MTM SA derivative of claim 5, wherein R₁ is proline (Pro), alanine (Ala), serine (Ser), cysteine (Cys), histidine (His), tryptophan (Trp), or a tyrosine (Tyr) conjugate.

7. A method of preparing the MTM SA derivative of claim 3, the method comprising coupling the *terminal carboxylic acid group* of MTM SA with an amine to form the MTM SA derivative.
8. A method of treating cancer or neuro-disease, the method comprising administering to a patient in need thereof an effective amount of the MTM SA derivative or a pharmaceutically acceptable salt thereof of any one of claims 1, 2, 3, 4, 5, or 6.
9. The method of claim 8, wherein the method comprises treating Ewing sarcoma.
10. The method of claim 8, wherein the method comprises treating lung cancer.
11. The method of claim 8, wherein the method comprises treating colon cancer.

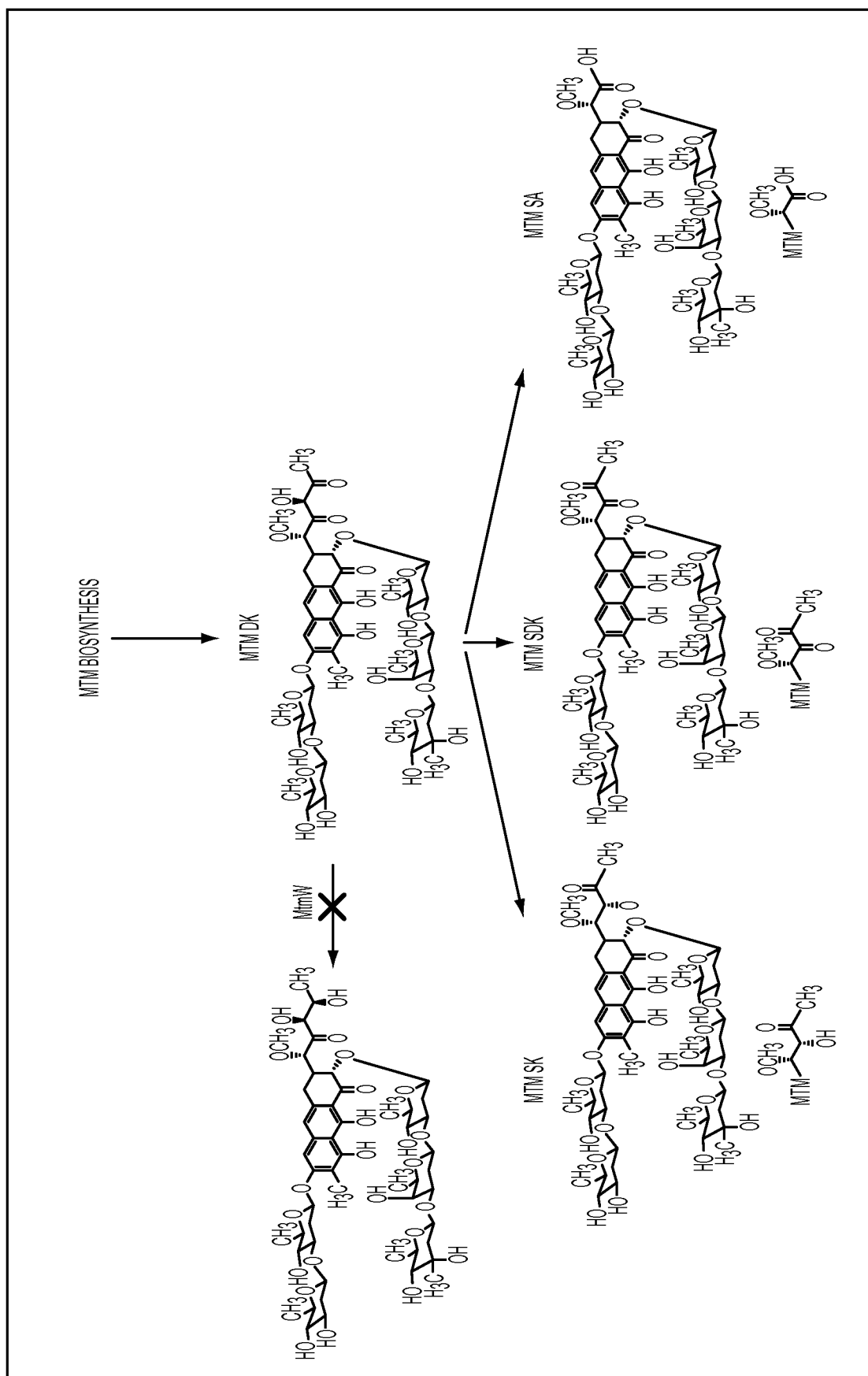


FIG. 1



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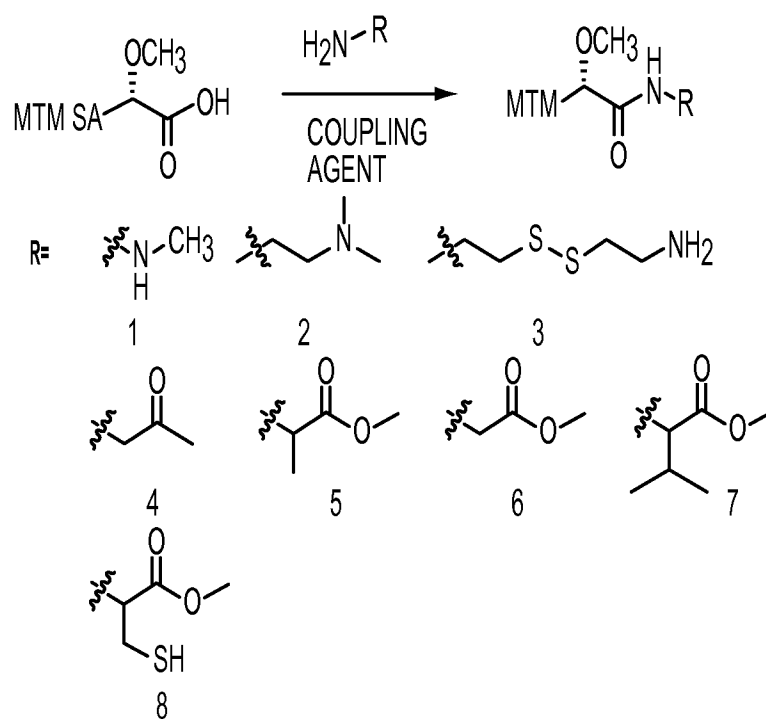


FIG. 3

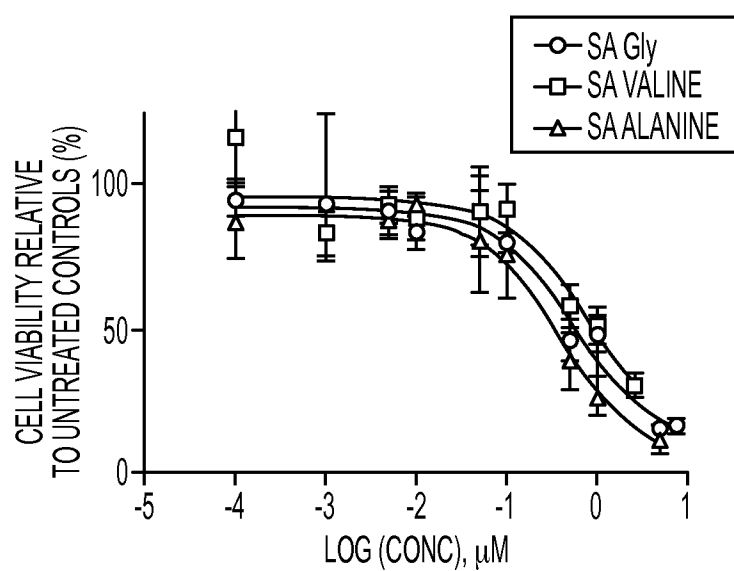


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/074725

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/704 (2014.01)

USPC - 514/33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 31/704; C07H 15/24 (2014.01)

USPC - 514/33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - A61K 31/704; C07 H15/24 (2014.02)

ECLA - A61K 31/704; C07 H15/24

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, STN, Google Scholar, PubChem

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0270823 A1 (NÚÑEZ GONZÁEZ et al) 25 October 2012 (25.10.2012) entire document	1, 8, 10, 11
A	HU et al. Chromomycin SA analogs from a marine-derived Streptomyces sp. Bioorganic and Medicinal Chemistry, 19(17): 5183-5189, 2011. [retrieved on 18 March 2014]. Retrieved from the Internet. <URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3159829/pdf/nihms313314.pdf >. entire document	1-11
A	GB 1 446 536 A (SELA et al) 18 August 1976 (18.08.1976) entire document	1-11

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Date of the actual completion of the international search

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Date of mailing of the international search report

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