(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number
WO 03/057687 A1

(51) International Patent Classification[7]: C07D 315/00, C12P 7/40, 7/24, 7/26

(21) International Application Number: PCT/US02/41604

(22) International Filing Date: 23 December 2002 (23.12.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/344,279 27 December 2001 (27.12.2001) US

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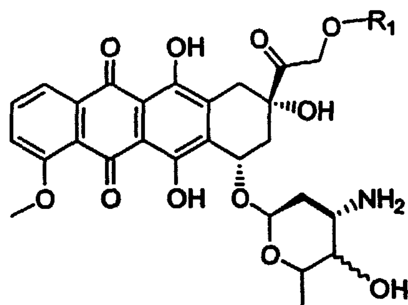
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),

[Continued on next page]

(54) Title: METHODS FOR PREPARING DOXORUBICIN DERIVATIVES



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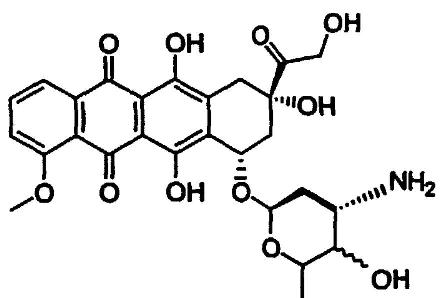

(II)

(57) Abstract: The present invetion relates to a process for preparation of a product compound of formula (I), where R_1 is an acyl group. The process involves reacting a starting compound of formula (II), with an activated acyl donor compound in the presence of a non-chemically modified lipase, under conditions effective to produce the product compound.

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Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS FOR PREPARING DOXORUBICIN DERIVATIVES

FIELD OF THE INVENTION

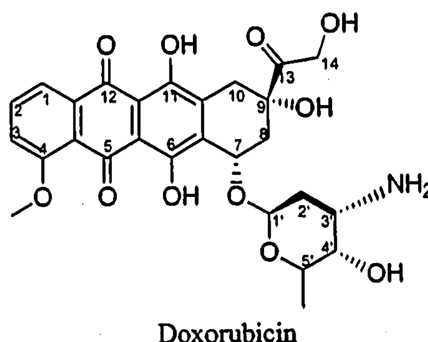
[0001] The present invention relates to methods for preparing derivatives of the anti-cancer compound doxorubicin that are site-selectively acylated at the C-14 position.

[0002] This work was supported by the U.S. Department of Energy's Office of Industrial Technologies, the National Science Foundation, Grant Nos. BES-9902878 and BES-9811352, and the National Institutes of Health, Grant No. 1R41 RR13512-01. The U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Over thirty years ago, the anthracycline doxorubicin was first isolated from *Streptomyces peucetius* var. *Caesius* and recognized for its antitumor properties (Arcamone et al., Biotechnology and Bioengineering, 11:1101-1110 (1969)). While doxorubicin has remained one of the most potent and extensively-used chemotherapeutics in cancer treatment (Hortobagyi, Drugs, 54:1-7 (1997); Lown, Pharmacol. Ther., 60:185-214(1993)), it has a number of therapeutic drawbacks including dose-limiting cardiotoxicity and susceptibility to multidrug resistance (Hortobagyi, Drugs, 54:1-7 (1997); Gewirtz, Biochem. Pharmacol., 57:727-741 (1999); Muggia et al., Crit. Rev. Oncol. Hematol., 11:43-64 (1991); Weiss, Semin. Oncol., 19:670-686 (1992)). In the intervening decades, thousands of doxorubicin analogs have been developed in the largely unsuccessful search for an improved pharmaceutical (Weiss, Semin. Oncol., 19:670-686 (1992)). As with many other natural compounds, the chemical synthesis of doxorubicin is a challenging process. Several extensive procedures exist for generating doxorubicin, but none is convenient for rapid generation of diverse analogs (Broadhurst et al., Tetrahedron, 40:4649-4656 (1984)). Most efforts, therefore, to modify doxorubicin take the more practical approach of starting from the complete drug. Unfortunately, the polyfunctional structure (including two

phenolic hydroxyls, and one each of a quinone, ketone, primary hydroxyl, secondary hydroxyl, tertiary hydroxyl, primary amine, methyl ether, and glycosidic linkage) and the sensitivity to pH, heat, metal ions, and light complicate conventional efforts at controlled derivatization (Bouma et al., Pharm. Weekbl [Sci], 8:109-133 (1986)).



Doxorubicin

[0004] The abundant literature on doxorubicin structure-activity relationships (Farquhar et al., Journal of Medicinal Chemistry, 41:965-972 (1998); Gabbay et al., Biochemistry, 15:2062-2070 (1976); Priebe et al., Pharmacol. Ther., 60:215-234 (1993); Chaires et al., Biochemistry, 35:2047-2053 (1996); Capranico et al., Molecular Pharmacology, 45:908-915 (1994)) provides some guidance for focusing analog design. Most notably, DNA intercalation is central to doxorubicin's primary mode of action: the inhibition of topoisomerase II and subsequent DNA strand breakage. When doxorubicin is intercalated, the C-9 ketone element and the daunosamine sugar project into the minor groove and stabilize the DNA complex by hydrogen bonding and by interacting with topoisomerase II. Although promising doxorubicin derivatives to date predominately involve alterations to the sugar structure (Farquhar et al., Journal of Medicinal Chemistry, 41:965-972 (1998); Capranico et al., Molecular Pharmacology, 45:908-915 (1994); Arcamone et al., Pharmacology & Therapeutics, 76:117-124 (1997)), such as the attachment of alkylating agents, a relatively small number of studies advocate the C-9 site as a promising target for activity-enhancing modifications (Scott et al., British Journal of Cancer, 53:595-600 (1986); Israel et al., Cancer Res., 35:1365-1368 (1975); Adams et al., Journal of Medicinal Chemistry, 33:2380-2384 (1990); Coley et al., Anti-Cancer Drug

Design, 7:471-481 (1992); Povarov et al., Bioorganicheskaya Khimiya, 21:925-932 (1995)).

[0005] The chemical fragility and functional diversity of doxorubicin provide an excellent opportunity to take advantage of biocatalysis for regioselective reaction under mild reaction conditions. Many of the original, and ongoing, efforts to develop anthracycline analogs have utilized microbial transformations (Marshall et al., Biochemistry, 15:4139-4145 (1976); Grafe et al., Biotechnol. Adv., 7:215-239 (1989); Johdo et al., J. Antibiot (Tokyo), 49:669-675 (1996)). But none of these allow selective modification of the 14-position. In addition, the heterogeneous reaction environment, significant potential for side reactions catalyzed by the mixture of enzymes present within the cell, and difficulties for isolation and purification of small molecules from aqueous microbial fermentation broths make the use of isolated enzymes in non-aqueous solvents more likely to be practical for a commercially-viable process.

[0006] Nonaqueous enzymology is an increasingly valuable tool for synthetic chemistry (Khmelnitsky et al., Curr. Opin. Chem. Biol., 3:47-53 (1999)). Within the unnatural environment of organic solvents, enzymes catalyze regioselective and enantioselective reactions under mild conditions with a broad range of substrates (Wong et al., Enzymes in Synthetic Organic Chemistry, 1st ed.; Tarrytown, N.Y., Oxford, U.K.:Pergamon (1994)); Drauz et al., Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook, Vch: Weinheim:New York (1995)). The derivatization of natural products and synthetic multifunctional substrates presents an especially rich opportunity for exploiting biocatalyst selectivity. By employing enzymatic techniques, chemists can often circumvent the challenges of protective chemistries that might be required to perform identical transformations using traditional synthetic methods. Hydrolytic enzymes can be used in organic solvents or biphasic environments to biocatalytically acylate nucleophilic groups. This procedure is most often applied to small compounds with high structural similarity to the enzymes' natural substrates. Biotransformation of novel complex structures has been a subject of increasing interest, especially as part of a biocatalytic approach to combinatorial chemistry (Altreuter et al., Current Opinion in Biotechnology, 10:130-136 (1999); Krstenansky et al., Bioorgan. Med. Chem., 7:2157-2162 (1999); Michels et al.

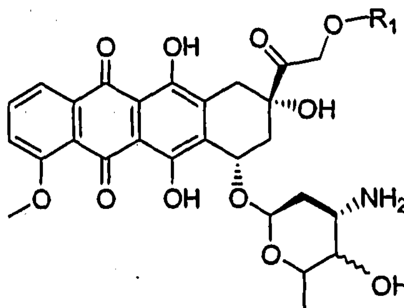
Trends Biotech., 16:210-215 (1998); Mozhaev et al., Tetrahedron, 54:3971-3982 (1998); Khmelnitsky et al., J. Amer. Chem. Soc., 119:11554-11555 (1997)).

[0007] Previously, the use of one chemically modified lipase for the acylation of doxorubicin has been presented (Altreuter et al., "Combinatorial Biocatalytic Derivatization of Doxorubicin," (abstract) American Chemical Society Meeting (1998)). Enzyme modifications found to allow catalytic activity with doxorubicin included solubilization of the enzyme in a enzyme-surfactant complex, and co-lyophilization of enzyme in a salt solution. The selectivity of the reaction relied on the type of modification applied to the enzyme, with different types of enzyme modification affording from one to three different acylated doxorubicin products upon reaction. In addition, these modification and solubilization techniques require a separate modification step to prepare the enzyme catalyst as well as a complicated removal step to remove either the modified enzyme or modification agent (e.g. surfactant) from the product stream.

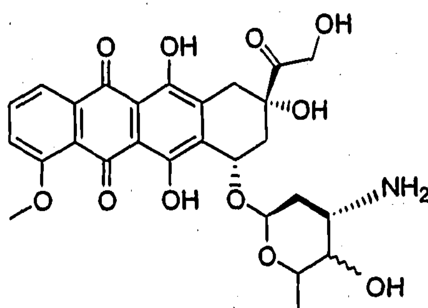
[0008] The present invention is directed to overcoming these deficiencies in the art.

SUMMARY OF THE INVENTION

[0009] The present invention relates to a process for preparation of a product compound of the formula:



where R_1 is an acyl group. The process involves reacting a starting compound of the formula:



with an activated acyl donor compound in the presence of a non-chemically modified lipase, under conditions effective to produce the product compound.

5 [0010] The present invention describes a process for the use of enzymes for selective 14-O-acylation of doxorubicin using acyl donor compounds such as vinyl esters, trihaloethyl esters, or vinyl carbonates. The 14-hydroxyl group is the third most activated site for chemical acylation, after the sugar amine and secondary hydroxyl. Therefore, chemical direct 14-O-acylation of doxorubicin

10 requires protection/deprotection of the 3'-NH_2 and 4'-OH moieties. Alternative chemical syntheses already known for the production of 14-acyl doxorubicin derivatives have involved an alkyl halide intermediate converted to the ester by a nucleophilic displacement. Both of these chemical routes are multistep, and give relatively low (<40%) overall yields. Therefore, the scheme of the present

15 invention represents an improved process over alternative chemical routes.

[0011] Furthermore, in comparison to the solubilized or salt-activated enzymes, the use of non-chemically modified enzymes removes the need for a modification step to activate the enzyme catalyst and simplifies product recovery by eliminating the need to remove either the enzyme or activating agent such as

20 Aerosol OT (AOT) from the resultant product stream.

[0012] In addition, the scheme of the present invention could be applied for the more efficient production of known 14-O-acyl doxorubicin derivatives of economic value currently on the market or in clinical development.

[0013] Accordingly, the regioselective acylation of doxorubicin using non-

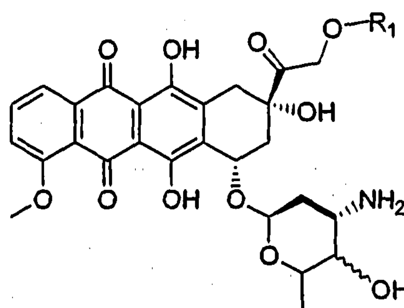
25 chemically modified lipases in a nonaqueous environment represents a unique pathway to achieve potent cytotoxic doxorubicin analogs, and expands the

repertoire of biocatalytic techniques available for a combinatorial lead-development program.

DETAILED DESCRIPTION OF THE INVENTION

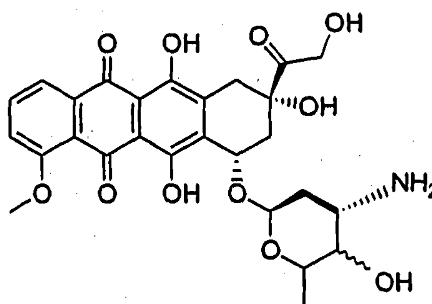
5

[0014] The present invention relates to a process for preparation of a product compound of the formula:



10

where R_1 is an acyl group. The process involves reacting a starting compound of the formula:



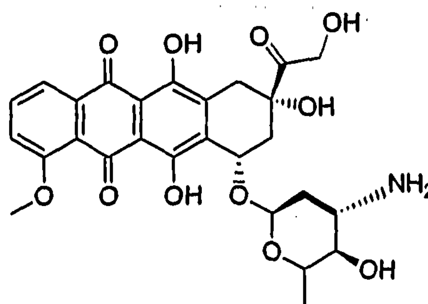
with an activated acyl donor compound in the presence of a non-chemically
15 modified lipase, under conditions effective to produce the product compound.

[0015] In order to obtain complete conversion of the starting compound to the acylated product compound, the above process can also be carried out under conditions effective to exclusively acylate the starting compound at its C-14 position.

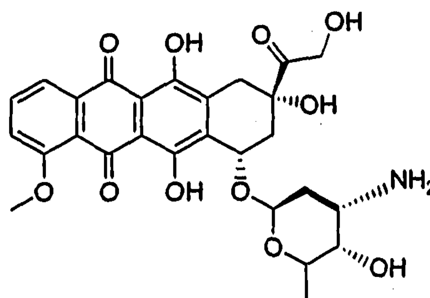
20 **[0016]** The reaction of the present invention can be carried out in an organic solvent. Possible organic solvents include but are not limited to toluene,

methyl-tert-butyl ether, pyridine, chloroform, acetonitrile, N,N-dimethyl formamide ("DMF"), tetrahydrofuran ("THF"), isooctane, and mixtures of these solvents.

[0017] Starting compounds that can be used for the present invention
5 include epirubicin, having the formula:



or doxorubicin, having the formula:



[0018] Different activated acyl donor compounds such as vinyl
10 carbonates, vinyl esters, or trihaloethyl esters can be used for the present invention.

[0019] Examples of vinyl carbonates include butyl vinyl carbonate; 1-methyl-3-piperidine methanol vinyl carbonate; 3,3'-diethoxypropanol vinyl carbonate; 4-tert-butylphenethyl vinyl carbonate; benzyl vinyl carbonate; 4-
15 methyl-5-thiazole ethanol vinyl carbonate; glycidol vinyl carbonate; 1,3-propylene divinyl carbonate; 1,4-cyclohexane dimethanol di(vinyl carbonate); 1,6-hexanediol di(vinyl carbonate); 4-hydroxybenzyl alcohol di(vinyl carbonate); 2,3-O-benzylidene thrietol di(vinylcarbonate); 2,5-furan-dimethanol di(vinylcarbonate); 2,6-pyridine dimethanol di(vinylcarbonate); acetone oxime
20 vinyl carbonate; 1,4-but-2-ene-diol di(vinylcarbonate); 3-thiophene methanol vinyl carbonate; 2-methylsulfonyl ethanol vinyl carbonate; 4-(2-hydroxyethyl)

morpholine vinyl carbonate; and 3-methyl-2-norbornane methanol vinyl carbonate.

[0020] Examples of vinyl esters include glyoxylic acid diethylacetal vinyl ester; 3,3-diphenyl propionic acid vinyl ester; 3,5-dibromo-4-hydroxybenzoic acid vinyl ester; 3-(2-furyl) acrylic acid vinyl ester; 3,4-(methylenedioxy) phenyl acetic acid vinyl ester; norbornane acetic acid vinyl ester; 2-thiophene acetic acid vinyl ester; 2,6-dimethoxy nicotinic acid vinyl ester; 3-indol butyric acid vinyl ester; pyrrole-2-carboxylic acid vinyl ester; 3,4-(methylenedioxy) cinnamic acid vinyl ester; 4-formylcinnamic acid vinyl ester; N-CBZ-isonipecotic acid vinyl ester; N-CBZ-L-proline vinyl ester; N-CBZ-tyrosine vinyl ester; N-CBZ-alanine vinyl ester; Fmoc-sarcosine vinyl ester (Fmoc-N-Me-Gly-OH vinyl ester); N-CBZ-Glycine vinyl ester; N-CBZ-phenylalanine vinyl ester; 2-furoic acid vinyl ester; acrylic acid vinyl ester; benzoic acid vinyl ester; bromoacetic acid vinyl ester; butyric acid vinyl ester (vinyl butyrate); caproic acid vinyl ester; chloroacetic acid vinyl ester; chloroformic acid vinyl ester; chlorobenzoic acid vinyl ester; cinnamic acid vinyl ester; crotonic acid vinyl ester; formic acid vinyl ester; iodoacetic acid vinyl ester; lauric acid vinyl ester; methacrylic acid vinyl ester; myristic acid vinyl ester; n-capric acid vinyl ester; n-caprylic acid vinyl ester; palmitic acid vinyl ester; phenyl acetic acid vinyl ester; pivalic acid vinyl ester; propionic acid vinyl ester; sorbic acid vinyl ester; stearic acid vinyl ester; acetic acid vinyl ester; 2-ethyl hexane vinyl ester; vinyl salicylate; trimethyl-vinyloxycarbonylmethyl-ammonium bromide; 3-amino-4,6-dimethyl-thieno [2,3-b] pyridine-2-carboxylic acid vinyl ester (1S-27086); 3-amino-6-thiophen-2-yl-4-trifluoromethyl-thieno [2,3-b] pyridine carboxylic acid vinyl ester (1S-21501); divinyl succinate; divinyl glutarate; divinyl adipate; divinyl sebacate; or divinyl subarate.

[0021] The trihaloethyl esters of the present invention can be trifluoroethyl esters or tricholroethyl esters, preferably trifluoroethyl esters. Examples of trifluoroethyl esters include glyoxylic acid diethylacetal trifluoroethyl ester; 3,3-diphenyl propionic acid trifluoroethyl ester; 3,6-dioxaheptanoic acid trifluoroethyl ester; oxalic acid trifluoroethyl ester; malonic acid trifluoroethyl ester; (-)-2-oxo-4-thiazolidine-2-carboxylic acid trifluoroethyl ester; pyrazine-2-carboxylic acid trifluoroethyl ester; nicotinic acid trifluoroethyl ester; 1,4-cyclohexane dicarboxylic acid di trifluoroethyl ester; terephtalic acid di trifluoroethyl ester; 4-

(dimethylamino) benzoic acid trifluoroethyl ester; 4-(bromomethyl) phenylacetic acid trifluoroethyl ester; benzimidazole propionic acid trifluoroethyl ester; Fmoc-L-thiazolidine-4-carboxylic acid trifluoroethyl ester; glutaric acid ditrifluoroethyl ester; 2-formylphenoxy acetic acid trifluoroethyl ester; 4-carboxybenzaldehyde trifluoroethyl ester; 4-(dimethylamino)phenyl acetic acid trifluoroethyl ester; isonitinic acid trifluoroethyl ester; or picolinic acid trifluoroethyl ester.

[0022] In another embodiment of the present invention, R_1 of the product compound is glyoxylic acid diethylacetal.

[0023] Non-chemically modified lipases of the present invention can either be native (wild-type) lipases or genetically-modified lipases engineered using standard methods of molecular biology (Berglund, "Controlling Lipase Enantioselectivity for Organic Synthesis," Biomol. Eng., 18(1): 13-22 (2001); Brocca et al., "Novel Lipases Having Altered Substrate Specificity, Methods for Their Preparation, and Their Use in Biocatalytic Applications," Eur. Pat. Appl. pp.33 (2001); Svendsen, "Lipase Protein Engineering," Biochim. Biophys. Acta, 1543(2): 223-238 (2000); Wong et al., "Lipase Engineering: A Window into Structure-Function Relationships," Methods in Enzymology, 284: 171-84 (1997), which are hereby incorporated in their entirety).

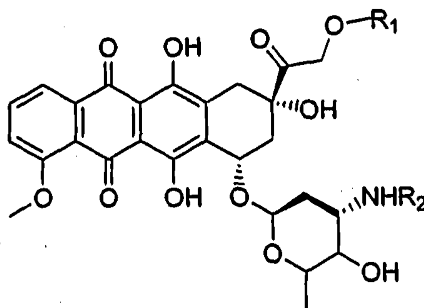
[0024] Non-chemically modified lipases that can be used for the selective 14-O-acylation of the starting compound include lipases from microbial sources such as *Alcaligenes* sp., *Candida cylindracea*, *Candida rugosa*, *Mucor javanicus*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Phycomyces nitens*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizopus arrhizus*, *Rhizopus delemar*, *Rhizopus niveus*, *Rhizopus oryzae*, or *Thermomyces* sp.

[0025] In one embodiment, the nonaqueous biocatalysis of the present invention can be carried out where the non-chemically modified lipase is immobilized to a solid support. Examples of solid support include diatomaceous earth, polypropylene, or acrylic resins. Such solid-phase synthesis has a number of advantages such as simplifying purification, enabling excess reagents to drive reactions, avoiding limitations of substrate solubility, and facilitating handling of reactions on a small scale.

[0026] In a typical reaction, doxorubicin (free base) is obtained by adding potassium tert-butoxide to commercially-available doxorubicin HCl salt dissolved

in THF. The recovered doxorubicin free base is then suspended in toluene. A suitable activated acyl donor is added, followed by the addition of a suitable unmodified lipase as a lyophilized powder or immobilized to a solid support. The reaction mixture is incubated at a fixed temperature (25-50°C), with orbital shaking, from 16 hours up to 2 days. Reaction progress is followed by thin layer chromatography ("TLC") and liquid chromatography/mass spectroscopy ("LC/MS"). Upon depletion of doxorubicin, the reaction mixture is filtered through filter paper to remove insoluble enzyme powder, and is concentrated under vacuum. Excess acyl donor is removed using a hexane wash and the 14-acyl-doxorubicin is purified using silica gel chromatography.

[0027] The above scheme of the present invention could also be applied for the more efficient production of known 14-O-acyl doxorubicin derivatives, such as simple 14-O-acyl derivatives of doxorubicin or the trifluoroacetamino analogs, including Valrubicin, AD-143, dextrorubicin, AD-198, galrubicin, ME-2303, as well as other compounds of biological interest. Thus, another aspect of the present invention relates to a process that involves further reacting the product compound (14-O-acyl doxorubicin derivative) with a chemical acylating agent under conditions effective to produce a second product compound of the formula:



where R_2 contains a carboxyl, a carbonyl, a sulfuryl, a phosphoryl, a thiocarbonyl, a xanthane, or a substituted or unsubstituted benzyl group.

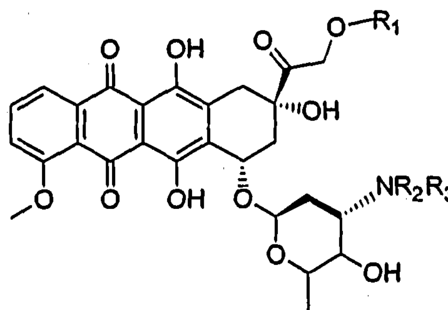
[0028] In one embodiment of this aspect of the present invention, R_1 is an acyl radical of acetic, octanoic, benzoic, propionic, phenylacetic, nicotinic, formic, butyric, glycolic, glycinic, succinic, 2'-hydroxy-naphthoic, cyclopentylpropionic, 2'-pyrrolcarboxylic, carbamic or ethylcarbonic acids, and R_2 is $COCF_3$.

[0029] In another embodiment, R_1 is an acyl radical of an alkanoate having 4 to 10 carbon atoms and R_2 is $COCF_3$. The alkanoate can be valerate, butanoate, isobutyrate, hexanoate, or octanoate.

[0030] In yet another embodiment, R_1 is an acyl radical of a dicarboxylic acid and R_2 is $COCF_3$. The dicarboxylic acid can be glutaric acid, adipic acid, subaric acid, or succinic acid.

[0031] In another embodiment, R_2 of the second product compound is a benzyl group. R_1 can be an acyl radical of an alkanoate having 4 to 17 carbon atoms, glutaric acid, adipic acid, subaric acid, succinic acid, acetic acid, octanoic acid, benzoic acid, propionic acid, phenylacetic acid, nicotinic acid, formic acid, butyric acid, glycolic acid, glycinic acid, succinic acid, 2'-hydroxy-naphthoic acid, cyclopentylpropionic acid, 2'-pyrrolcarboxylic acid, carbamic acid or ethylcarbonic acid. Preferably, R_1 is an acyl radical of a valerate or glutaric acid.

[0032] Another aspect of the present invention relates to a process that involves further reacting the second product compound (14-O-acylated doxorubicin derivative which is also acylated at the 3'-NH_2 position) with a chemical alkylating agent under conditions effective to produce a third product compound of the formula:



where R_3 contains a substituted or unsubstituted C_1-C_{10} alkyl or benzyl group.

[0033] In one embodiment of this aspect of the present invention, R_1 is an acyl radical of acetic, octanoic, benzoic, propionic, phenylacetic, nicotinic, formic, butyric, glycolic, glycinic, succinic, 2'-hydroxy-naphthoic, cyclopentylpropionic, 2'-pyrrolcarboxylic, carbamic or ethylcarbonic acids, and R_2 is $COCF_3$.

[0034] In another embodiment, R_1 is an acyl radical of an alkanoate having 4 to 10 carbon atoms and R_2 is $COCF_3$. The alkanoate can be valerate, butanoate, isobutyrate, hexanoate, or octanoate.

[0035] In yet another embodiment, R_1 is an acyl radical of a dicarboxylic acid and R_2 is $COCF_3$. The dicarboxylic acid can be glutaric acid, adipic acid, subaric acid, or succinic acid.

[0036] In another embodiment, R_2 of the third product compound is a benzyl group. R_1 can be an acyl radical of an alkanoate having 4 to 17 carbon atoms, glutaric acid, adipic acid, subaric acid, succinic acid, acetic acid, octanoic acid, benzoic acid, propionic acid, phenylacetic acid, nicotinic acid, formic acid, butyric acid, glycolic acid, glycinic acid, succinic acid, 2'-hydroxy-naphthoic acid, cyclopentylpropionic acid, 2'-pyrrolcarboxylic acid, carbamic acid or ethylcarbonic acid. Preferably, R_1 is an acyl radical of a valerate or glutaric acid.

EXAMPLES

[0037] The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1 – Preparation of Doxorubicin Free Base

[0038] Doxorubicin HCl (100 mg) was suspended in 20 mL anhydrous THF. While stirring, 1 equivalent of potassium *tert*-butoxide (172 μL of 1.0 M solution in THF, 0.17 mmol) was slowly added. An immediate color change from red to violet was observed. The reaction was stirred for ca. 1 hr at room temperature, during which time the color slowly turned from violet to dark red. Doxorubicin free base was recovered by removing THF under a stream of argon.

Example 2 - Synthesis of Doxorubicin-14-Butyrate

[0039] 100 mg of doxorubicin (free base) was suspended in toluene (85 mL). 50 mM of vinyl butyrate (135 μL) was added, followed by ca. 2 g of lipase PS30 (Amano, Lombard, IL). The reaction mixture was incubated at 45°C (250 rpm) for 24 hr. Reaction progress was followed by TLC and LC/MS. Upon

depletion of doxorubicin, the reaction mixture was filtered with filter paper and concentrated under vacuum. Excess acyl donor was removed using a hexane wash (2x25 mL). Silica gel chromatography (eluent 6:1 $CHCl_3$:MeOH) gave doxorubicin-14-butyrate (44 mg, 41%) as a red solid in >98% purity.

5

Example 3 – Synthesis of Doxorubicin-14-Adipate

[0040] Doxorubicin hydrochloride (110 mg, 0.19 mmol) was dissolved in nanopure water (50 mL), and chloroform (10 mL) was added. The aqueous phase
10 was titrated with triethylamine to the point when the color changed to violet. After thorough agitation of the mixture, the orange chloroform phase was collected, and the extraction was repeated with new portions of chloroform (10 mL), until the aqueous phase was nearly colorless. The chloroform fractions were combined, dried over Na_2SO_4, and evaporated to dryness. This amount was
15 then suspended in a mixture of anhydrous toluene (70 mL), chloroform (20 mL), and divinyl adipate (0.71 g, 3.6 mmol, TCI). 500 mg each of lipases AK and PS30 (Amano, Lombard, IL), both immobilized on Accurel (Akzo Nobel, Chicago, IL) were added to start the reaction. The mixture was shaken at ca. 250 rpm at 45°C for 6 days, after which ca. 95% conversion of doxorubicin to a
20 mixture of products was observed by LC/MS sampling. The enzyme was removed by filtration, and the retained solids were washed with chloroform (10 mL) until the wash was nearly colorless. The collected washes were combined with the filtrate and the solvent removed under vacuum to yield an oil. The oil was then washed 6 times with hexane (10 mL) and dried to yield 167.8 mg
25 of a red solid. 8.4 mg of doxorubicin-14-vinyl adipate was isolated by reversed-phase HPLC (Shimadzu LC-8A, Columbia, MD).

Example 4 –Purification of Doxorubicin-14-Esters

30 *Doxorubicin-14-Esters*

[0041] The isolation of doxorubicin14-esters involved converting the doxorubicin related material into an HCl salt followed by separation on reversed-phase HPLC (Shimadzu LC-8A, Columbia, MD). The reaction mixture was dissolved in a small amount of chloroform and added to a 0.1 M solution of HCl

or trifluoroacetic acid in anhydrous ether at 0°C. The precipitated solids were recovered by centrifugation, washed with ether and dried under vacuum.

[0042] The resulting water-soluble solids, indicating that the material is in the salt form, were purified on a LC-8A Shimadzu preparative chromatograph equipped with a Symmetry Shield RP8 (7 µm, 50x200 mm, Waters) a gradient mobile phase of water (+ 0.01% TFA) and acetonitrile (+ 0.01% TFA) at 64.8 mL/min. Fractions were pooled based upon HPLC analysis and the resulting product-containing solution was dried under vacuum to give isolated product as a red solid.

Alternative Method of Doxorubicin-14-Ester Purification

[0043] A polystyrene/divinylbenzene column (1 cm x 6.5 cm; Diaion HP20ss, Supelco, Bellefonte, PA) was conditioned with methanol and equilibrated to 0.2% TEA-acetate in 15% acetonitrile/water. Flow rate was 0.4 column volumes (CV)/min; all mobile phases contained 0.2% TEA-acetate. Acetylated doxorubicin reaction product (approx. 12 mg) was solution loaded and eluted in step gradient fashion from 25% to 35% acetonitrile/water at 5% increments of 6 to 9 CV each. The column was washed with methanol. Fractions were collected in 2 CV increments.

Example 5 – Confirmation of Product Structure

LC/MS analysis of Doxorubicin Derivatives.

[0044] Doxorubicin and its derivatives exhibit ionization as M+H (positive mode) and M–H (negative mode). Doxorubicin that is not derivatized on the adriamycinone subunit also shows a characteristic fragment of 397 amu (positive mode) and 395 amu (negative mode). Doxorubicin 14-acyl derivatives exhibit an increase in molecular weight of the adriamycinone fragment depending upon the mass of the acyl unit.

[0045] Samples were analyzed on a PE-Sciex API 2000 LC/MS/MS system equipped with an HP1000 photodiode array detector (doxorubicin has characterisic peaks at 234, 254, 292 and 496 nm). A Luna C8 (5µ, 50 x 2 mm,

Phenomenex, Torrance, CA) column was used with a mobile phase gradient of water (+ 0.4% HOAc) and acetonitrile (+ 0.4% HOAc).

NMR Analysis of Doxorubicin Derivatives.

5 **[0046]** The formation of 14-acyl derivatives is clearly demonstrated in the NMR spectrum by a downfield shift of the C-14 methylene protons. They appear as two doublets between 5 and 5.5 ppm in deuterated acetone or methanol. Samples were run on a WM-360 MHz or a DRX-400 MHz (Bruker, Billerica, MA) spectrophotometer. Doxorubicin trifluoroacetamide derivatives were 10 analyzed from deuterated acetone and doxorubicin salt derivatives from deuterated methanol.

<u>Example 6</u> – Identification of Enzymes for Selective 14-O-Acylation

15 **[0047]** Doxorubicin free base (2 mM) was dissolved in toluene containing 50 mM of vinyl butyrate (TCI, Portland OR), and 0.5 mL was added to a each well of a 96-well plate (2 mL total volume of each well), each well prefilled with ca. 100 mg of a different lipase as a dry solid. The plate was sealed and incubated at 45°C and rotated at 10 rpm in a custom incubator for up to 5d. Samples were 20 periodically withdrawn and analyzed by high-throughput LC/MS to determine the product identity and best conversion to 14-butyryl-doxorubicin.

[0048] With vinyl butyrate as the activated acyl donor, the lipases from *Pseudomonas cepacia* (PS30, Amano, Lombard, IL) and *Pseudomonas fluorescens* (AK, Amano, Lombard, IL) were identified as the most efficient 25 catalysts. However, when using other acyl donors of interest, such as vinyl benzoate or glutaric acid ditrifluoroethyl ester, different enzymes were found to be the most efficient catalysts for the desired reaction (Table 1). In general, reactivity with a broad set of acyl moieties of interest for commercial applications required a set of different lipases for the most efficient reaction, and sometimes to 30 detect any reaction at all with the particular activated acyl donor of interest. Therefore, it is best to perform a complete lipase screen with each acyl donor.

Table 1. Set of Non-Chemically Modified Lipase Enzymes for Selective Doxorubicin-14-O-Acylation

Lipase Trade Name	Source	Vinyl Benzoate	Glutaric acid diTFEE	Divinyl Adipate
AL	*Alcaligenes* sp.			•
Chirazyme L-10	*Alcaligenes* sp.		•	
PL	*Alcaligenes* sp.	•		•
QL	*Alcaligenes* sp.			•
OF	*Candida cylindracea*	•		•
AY-30	*Candida rugosa*	•		•
Chirazyme L-3	*Candida rugosa*	•	•	
type VII	*Candida rugosa*	•		•
"MJ"	*Mucor javanicus*	•		•
Lipase M-10	*Mucor javanicus*	•		
"PR"	*Penicillium roqueforti*			•
R-10	*Penicillium roqueforti*	•		•
Lipase PN	*Phycomyces nitens*	•	•	
AH	*Pseudomonas cepacia*		•	
LP "Amano" S	*Pseudomonas cepacia*	•	•	
PS-30	*Pseudomonas cepacia*	•		•
"LI"	*Pseudomonas* sp.	•		
AK	*Pseudomonas* sp.	•		•
Chirazyme L-6	*Pseudomonas* sp.	•	•	
"RA"	*Rhizopus arrhizus*	•		•
D	*Rhizopus delemar*		•	•
N	*Rhizopus niveus*	•	•	
Chirazyme L-8	*Thermomyces* sp.	•	•	

[0049] Twenty-four lipases were identified as having some activity for the selective 14-O-acylation of doxorubicin with at least one activated acyl donor in toluene.

<u>Example 7</u> – Rapid Selection of Enzymes and Optimization of Reaction Conditions for a Given 14-O-Acyl Doxorubicin Product

Rapid Selection of Enzymes

[0050] To maximize the utility and breadth of the invention, a rapid method for selecting the best biocatalyst for reaction with a given acyl donor was required. Doxorubicin free base (2 mM) was dissolved in toluene containing

50 mM of the selected activated acyl donor and 0.5 mL was added to a 96-well plate (2 mL total volume of each well), each well prefilled with ca. 100 mg of a different lipase as a dry solid. The plate was sealed and incubated at 45°C and rotated at 10 rpm in a custom incubator for 5d. Samples were periodically withdrawn and analyzed by high-throughput LC/MS to determine best conversion to the desired 14-O-ester.

Rapid Optimization of Reaction Conditions

[0051] To maximize the efficiency of each reaction, ca. 100 mg of the best biocatalyst for a desired reaction was added in each well of a 96-well plate (2 mL total volume of each well). The selection of improved reaction conditions involved screening a variety of solvents and differently activated acyl donors for the transesterification of doxorubicin catalyzed by the non-chemically modified lipases at 45°C. Six neat solvents and two binary solvent mixtures were typically tested. Doxorubicin free base (2 mM) and activated acyl donor (50 mM) were dissolved in different organic solvents and added to separate wells. The plate was sealed and incubated at 45°C and rotated at 10 rpm in a custom incubator for up to 5d. Samples were periodically withdrawn and analyzed by high-throughput LC/MS to determine best conversion to the desired 14-O-acyl doxorubicin.

[0052] For instance, lipase PS30 was able to catalyze the desired 14-O-acylation reaction with vinyl esters in four solvent systems: *tert*-butyl methyl ether ("MTBE"), toluene, 50% (v/v) pyridine in MTBE, and 50% (v/v) pyridine in toluene. Although MTBE was preferable due to a faster reaction rate and a lower boiling point, the solubility of doxorubicin free base was significantly lower in MTBE (0.5 mg/mL) than in toluene (2 mg/mL). Therefore, toluene was selected for the reactions.

[0053] The use of vinyl esters in solvents that were not rigorously dry led to the formation of an undesired side product. The enzyme-catalyzed hydrolysis of a vinyl ester produces acetaldehyde which can react with the 3'-NH_2 to generate a Schiff base. Using anhydrous solvents or other activated acyl donors minimized/eliminated the production of this side product.

Example 8 – Use of Enzyme Immobilization Support

[0054] Certain immobilization supports, including Accurel (Akzo Nobel, Chicago, IL) (polypropylene beads), adsorb doxorubicin. This adsorption was mostly reversible, but methanol (which is a nucleophile in the alcoholysis of the newly formed ester) was the only solvent that was capable of removing doxorubicin from the Accurel beads. The support used for Chirazyme L-2, C2 (Biocatalytics, Pasadena, CA) (Novozyme 435 (Novozymes A/S, Bagsvaerd, Denmark)) also adsorbed doxorubicin, and this adsorption was at least partially irreversible. In some cases, switching to the free enzyme could alleviate this adsorption problem. This solution, however, has two drawbacks: first, some enzymes, such as AK, showed little or no activity when not immobilized. Second, free enzymes, at least in the case of PS30, were not as active as their immobilized forms.

[0055] Diatomaceous earth (Celite® filter agent (Hyflo Super Cel, Sigma, St. Louis, MO)) was tested as an alternate support. Although little or no doxorubicin adsorption was observed (on a small scale), the activity per mg of immobilized enzyme was lower than that of the Accurel-immobilized enzyme or the free enzyme. The unit activity on an enzyme weight basis, however, was comparable to the other supports.

Example 9 – N-(Trifluoroacetyl)-Doxorubicin-14-Butyrate Synthesis

[0056] 100 mg of doxorubicin (free base) was suspended in toluene and 50 mM of vinyl butyrate (135 μL) was added, followed by ca. 2 g of lipase PS30. The reaction mixture was incubated at 45 °C (250 rpm) for 24 hr. The reaction progress was monitored by TLC and liquid chromatography/mass spectroscopy ("LC/MS"). Upon the complete consumption of the doxorubicin, the reaction mixture was clarified through filter paper and the filtrate was cooled to 0°C. A magnetic stir bar was added and 250 μL of triethylamine was charged in one portion. To this chilled, stirred solution was added 180 μL of trifluoroacetic anhydride (1.5 equivalents) and the resulting suspension was allowed to warm to 25°C, overnight. Upon the complete consumption of the doxorubicin-14-butyrate, the reaction mixture was quenched with water. The layers were separated and the

organic layer was twice washed with water, dried and concentrated under vacuum. The resulting solid was suspended in hexane and filtered. Silica gel chromatography of the solid residue (gradient elution of EtOAc/hexanes 2:1 to EtOAc) afforded N-(trifluoroacetyl)-doxorubicin-14-butyrate as a red solid
5 (69 mg, 53% overall yield).

Example 10 – N-Benzyl-Doxorubicin-14-Butyrate Synthesis

[0057] 100 mg of doxorubicin (free base) was suspended in toluene and
10 50 mM of vinyl butyrate (135 μL) was added, followed by ca. 2 g of lipase PS30. The reaction mixture was incubated at 45°C (250 rpm) for 24 hr. The reaction progress was monitored by TLC and liquid chromatography/mass spectroscopy ("LC/MS"). Upon the complete consumption of the doxorubicin, the reaction mixture was clarified through filter paper. A magnetic stir bar was added and
15 20 μL of freshly distilled benzaldehyde was charged in one portion. This mixture was stirred at 26°C for 1 h at which time, a 1M solution of sodium cyanoborohydride in THF (200 μL) was added. Stirring was continued for 2 h. Water was added and the layers were separated. The aqueous layer was extracted with chloroform. The combined organic layers were evaporated to dryness and
20 the residue slurried in hexane. The solids were collected by filtration. Silica gel chromatography (eluent: chloroform with 1% methanol) afforded N-benzyl-doxorubicin-14-butyrate (91 mg, 72% overall yield).

Example 11 – N-Benzyl-N-(Trifluoroacetyl)-Doxorubicin-14-Butyrate
25 **Synthesis**

[0058] 100 mg of doxorubicin (free base) was suspended in toluene and 50 mM of vinyl butyrate (135 μL) was added, followed by ca. 2 g of lipase PS30. The reaction mixture was incubated at 45°C (250 rpm) for 24 hr. The reaction
30 progress was monitored by TLC and liquid chromatography/mass spectroscopy ("LC/MS"). Upon the complete consumption of the doxorubicin, the reaction mixture was clarified through filter paper. A magnetic stir bar was added and 20 μL of freshly distilled benzaldehyde was charged in one portion. This mixture was stirred at 26°C for 1 h at which time, a 1 M solution of sodium

cyanoborohydride in THF (200 μL) was added. Stirring was continued for 2 h. Water was added and the layers were separated. The aqueous layer was extracted with chloroform. The combined organic layers were dried over anhydrous sodium sulfate and clarified. A magnetic stir bar was added to the filtrate and the solution was cooled to 0°C in an ice/water bath. Triethylamine (200 μL) was charged in one portion. To this chilled, stirred solution was added 135 μL of trifluoroacetic anhydride and the resulting solution was allowed to warm to 25°C, overnight. Upon the complete consumption of the N-benzyl-doxorubicin-14-butyrate, the reaction mixture was evaporated to dryness. The mixture was partitioned between chloroform and water and mixed. The layers were separated and the organic layer was twice washed with water, dried and concentrated under vacuum. The resulting solid was suspended in hexane and filtered. Silica gel chromatography of the solid residue (gradient eluent: chloroform to 2% methanol in chloroform) afforded N-benzyl-N-(trifluoroacetyl)-doxorubicin-14-butyrate as a red solid (61 mg, 42% overall yield).

Example 12 – **N, N-Dibenzyl-Doxorubicin-14-Butyrate Synthesis**

[0059] 100 mg of doxorubicin (free base) was suspended in toluene and 50 mM of vinyl butyrate (135 μL) was added, followed by ca. 2 g of lipase PS30. The reaction mixture was incubated at 45°C (250 rpm) for 24 hr. The reaction progress was monitored by TLC and liquid chromatography/mass spectroscopy ("LC/MS"). Upon the complete consumption of the doxorubicin, the reaction mixture was clarified through filter paper. A magnetic stir bar was added and 20 μL of freshly distilled benzaldehyde was charged in one portion. This mixture was stirred at 26°C for 1 h at which time, a 1M solution of sodium cyanoborohydride in THF (200 μL) was added. Stirring was continued for 2 h. Water was added and the layers were separated. The aqueous layer was extracted with chloroform. The combined organic layers were evaporated to dryness and the residue slurried in hexane. The solids were collected by filtration and dissolved in chloroform. Benzyl bromide (21 μL) was added in one portion and the solution was stirred overnight at room temperature. Following evaporation of the solution, the residue was purified by silica gel chromatography (eluent:

chloroform with 1% methanol) afforded N, N-dibenzyl-doxorubicin-14-butyrate (84 mg, 59 % overall yield).

Example 13 - Doxorubicin-14-Valerate Synthesis

5

[0060] 130 mg of doxorubicin (free base) was dissolved in dry methyl-*tert*-butyl ether and 10 mmol of vinyl valerate (1.3 g) was added, followed by 2.5 g (85,000 U) of lipase PS30. The reaction mixture was incubated at 45°C (250 rpm) for 15 h. The reaction was monitored by TLC and liquid

10 chromatography mass spectroscopy ("LC/MS"). Upon complete consumption of the doxorubicin, the mixture was cooled to room temperature and clarified through filter paper. The filtrate was evaporated to dryness and the residue was resolved in dry THF (80 mL), divided into two equal parts and carried forward.

15 **Example 14 - N-(Trifluoroacetyl)-Doxorubicin-14-Valerate Synthesis**

[0061] To half of the fresh THF solution of doxorubicin-14-valerate (prepared above in Example 12) was added a magnetic stir bar and the solution was cooled to 0°C. To this chilled solution was added 250 µL of triethylamine,

20 followed by the slow addition of 120 µL of trifluoroacetic anhydride (1.5 equivalents). The resulting solution was allowed to warm to 25°C overnight. Upon the complete consumption of the doxorubicin-14-valerate, the slurry was concentrated under reduced pressure to a red solid. The residue was suspended in ethyl acetate and washed with water, 5% aqueous sodium bicarbonate solution,

25 dried and concentrated under vacuum. Silica gel chromatography of the solid residue (gradient elution of EtOAc/hexane 2:1 to EtOAc) afforded N-(trifluoroacetyl)-doxorubicin-14-valerate as a red solid (50 mg, 62% overall yield).

30 **Example 15 - N-Benzyl-Doxorubicin-14-Valerate Synthesis**

[0062] To the remaining half of the THF solution of doxorubicin-14-valerate was added a magnetic stir bar. To this stirred solution was added 20 µL of freshly distilled benzaldehyde and the solution was stirred at 24°C for 2 h. A

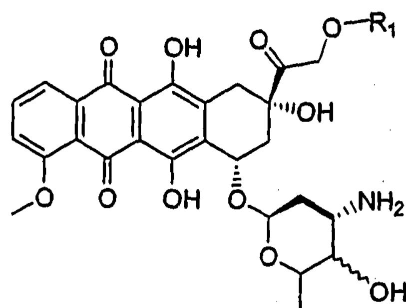
35 1 M solution of sodium cyanoborohydride in THF (130 µL) was added and the

reaction mixture was stirred for an additional 1h. Water was added and the organic solvent was evaporated under reduced pressure. The aqueous suspension was extracted with chloroform. The combined chloroform extracts were dried and evaporated to a minimal volume. Silica gel chromatography (eluent: 1% methanol
5 in chloroform) afforded N-benzyl doxorubicin-14-valerate as a red solid (43 mg, 52% overall yield).

[0063] Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from
10 the spirit and scope of the invention which is defined by the following claims.

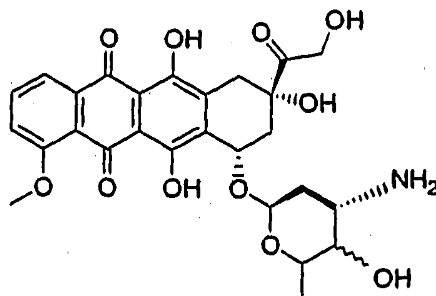
WHAT IS CLAIMED:

1. A process for preparation of a product compound of the formula:



wherein:
R_1 is an acyl group, said process comprising:

reacting a starting compound of the formula:



with an activated acyl donor compound in the presence of a non-chemically modified lipase, under conditions effective to produce the product compound.

2. The process of claim 1, wherein said reacting is carried out under conditions effective to exclusively acylate the starting compound at its C-14 position.

3. The process of claim 1, wherein said reacting is carried out in an organic solvent.

4. The process of claim 3, wherein the organic solvent is toluene or methyl-*tert*-butyl ether.

5. The process of claim 1, wherein the activated acyl donor compound is a vinyl carbonate, a vinyl ester, or a trihaloethyl ester.

6. The process of claim 5, wherein the activated acyl donor compound is a vinyl carbonate selected from the group consisting of: butyl vinyl carbonate; 1-methyl-3-piperidine methanol vinyl carbonate; 3,3'-diethoxypropanol vinyl carbonate; 4-tert-butylphenethyl vinyl carbonate; benzyl vinyl carbonate; 4-methyl-5-thiazole ethanol vinyl carbonate; glycidol vinyl carbonate; 1,3-propylene divinyl carbonate; 1,4-cyclohexane dimethanol di(vinyl carbonate); 1,6-hexanediol di(vinyl carbonate); 4-hydroxybenzyl alcohol di(vinyl carbonate); 2,3-O-benzylidene thrietol di(vinylcarbonate); 2,5-furan-dimethanol di(vinylcarbonate); 2,6-pyridine dimethanol di(vinylcarbonate); acetone oxime vinyl carbonate; 1,4-but-2-ene-diol di(vinylcarbonate); 3-thiophene methanol vinyl carbonate; 2-methylsulfonyl ethanol vinyl carbonate; 4-(2-hydroxyethyl) morpholine vinyl carbonate; and 3-methyl-2-norbornane methanol vinyl carbonate.

7. The process of claim 5, wherein the activated acyl donor compound is a vinyl ester selected from the group consisting of: glyoxylic acid diethylacetal vinyl ester; 3,3-diphenyl propionic acid vinyl ester; 3,5-dibromo-4-hydroxybenzoic acid vinyl ester; 3-(2-furyl) acrylic acid vinyl ester; 3,4-(methylenedioxy) phenyl acetic acid vinyl ester; norbornane acetic acid vinyl ester; 2-thiophene acetic acid vinyl ester; 2,6-dimethoxy nicotinic acid vinyl ester; 3-indol butyric acid vinyl ester; pyrrole-2-carboxylic acid vinyl ester; 3,4-(methylenedioxy) cinnamic acid vinyl ester; 4-formylcinnamic acid vinyl ester; N-CBZ-isonipecotic acid vinyl ester; N-CBZ-L-proline vinyl ester; N-CBZ-tyrosine vinyl ester; N-CBZ-alanine vinyl ester; Fmoc-sarcosine vinyl ester (Fmoc-N-Me-Gly-OH vinyl ester); N-CBZ-Glycine vinyl ester; N-CBZ-phenylalanine vinyl ester; 2-furoic acid vinyl ester; acrylic acid vinyl ester; benzoic acid vinyl ester; bromoacetic acid vinyl ester; butyric acid vinyl ester (vinyl butyrate); caproic acid vinyl ester; chloroacetic acid vinyl ester; chloroformic acid vinyl ester; chlorobenzoic acid vinyl ester; cinnamic acid vinyl ester; crotonic acid vinyl ester; formic acid vinyl ester; iodoacetic acid vinyl ester; lauric acid vinyl ester; methacrylic acid vinyl ester; myristic acid vinyl ester; n-capric

acid vinyl ester; n-caprylic acid vinyl ester; palmitic acid vinyl ester; phenyl acetic acid vinyl ester; pivalic acid vinyl ester; propionic acid vinyl ester; sorbic acid vinyl ester; stearic acid vinyl ester; acetic acid vinyl ester; 2-ethyl hexane vinyl ester; vinyl salicylate; trimethyl-vinyloxycarbonylmethyl-ammonium bromide; 3-amino-4,6-dimethyl-thieno [2,3-b] pyridine-2-carboxylic acid vinyl ester (1S-27086); 3-amino-6-thiophen-2-yl-4-trifluoromethyl-thieno [2,3-b] pyridine carboxylic acid vinyl ester (1S-21501); divinyl succinate; divinyl glutarate; divinyl adipate; divinyl sebacate; and divinyl subarate.

8. The process of claim 5, wherein the activated acyl donor compound is a trifluoroethyl ester or a trichloroethyl ester.

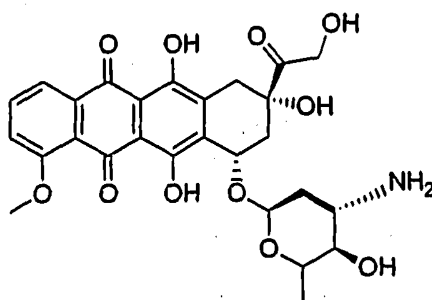
9. The process of claim 8, wherein the activated acyl donor compound is a trifluoroethyl ester selected from the group consisting of: glyoxylic acid diethylacetal trifluoroethyl ester; 3,3-diphenyl propionic acid trifluoroethyl ester; 3,6-dioxaheptanoic acid trifluoroethyl ester; oxalic acid trifluoroethyl ester; malonic acid trifluoroethyl ester; (-)-2-oxo-4-thiazolidine-2-carboxylic acid trifluoroethyl ester; pyrazine-2-carboxylic acid trifluoroethyl ester; nicotinic acid trifluoroethyl ester; 1,4-cyclohexane dicarboxylic acid di trifluoroethyl ester; terephtalic acid di trifluoroethyl ester; 4-(dimethylamino) benzoic acid trifluoroethyl ester; 4-(bromomethyl) phenylacetic acid trifluoroethyl ester; benzimidazole propionic acid trifluoroethyl ester; Fmoc-L-thiazolidine-4-carboxylic acid trifluoroethyl ester; glutaric acid ditrifluoroethyl ester; 2-formylphenoxy acetic acid trifluoroethyl ester; 4-carboxybenzaldehyde trifluoroethyl ester; 4-(dimethylamino)phenyl acetic acid trifluoroethyl ester; isonitinic acid trifluoroethyl ester; and picolinic acid trifluoroethyl ester.

10. The process of claim 1, wherein the non-chemically modified lipase is from a microbial source selected from the group consisting of *Alcaligenes* sp., *Candida cylindracea*, *Candida rugosa*, *Mucor javanicus*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Phycomyces nitens*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizopus arrhizus*, *Rhizopus delemar*, *Rhizopus niveus*, *Rhizopus oryzae*, and *Thermomyces* sp.

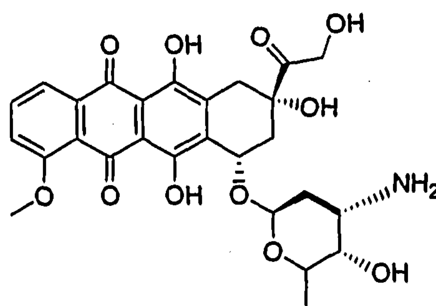
11. The process of claim 1, wherein the non-chemically modified lipase is immobilized to a solid support.

12. The process of claim 11, wherein the solid support is selected from the group consisting of diatomaceous earth, polypropylene, and acrylic resins.

13. The process of claim 1, wherein the starting compound has the formula:



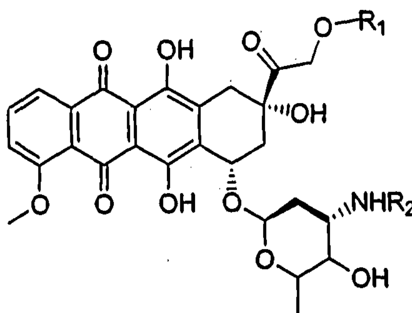
14. The process of claim 1, wherein the starting compound has the formula:



15. The process of claim 1, wherein R_1 is glyoxylic acid diethylacetal.

16. The process of claim 1 further comprising:

reacting the product compound with a chemical acylating agent under conditions effective to produce a second product compound of the formula:



where R_2 contains a carboxyl, a carbonyl, a sulfuryl, a phosphoryl, a thiocarbonyl, a xanthane, or a substituted or unsubstituted benzyl group.

17. The process of claim 16, wherein R_1 is an acyl radical of an acid selected from the group consisting of acetic, octanoic, benzoic, propionic, phenylacetic, nicotinic, formic, butyric, glycolic, glycinic, succinic, 2'-hydroxy-naphthoic, cyclopentylpropionic, 2'-pyrrolcarboxylic, carbamic and ethylcarbonic acids and R_2 is $COCF_3$.

18. The process of claim 16, wherein R_1 is an acyl radical of an alkanoate having 4 to 10 carbon atoms and R_2 is $COCF_3$.

19. The process of claim 18, wherein the alkanoate is selected from the group consisting of valerate, butanoate, isobutyrate, hexanoate, and octanoate.

20. The process of claim 19, wherein the alkanoate is valerate.

21. The process of claim 16, wherein R_1 is an acyl radical of a dicarboxylic acid and R_2 is $COCF_3$.

22. The process of claim 21, wherein the dicarboxylic acid is selected from the group consisting of glutaric acid, adipic acid, subaric acid, sebacic acid, and succinic acid.

23. The process of claim 22, wherein R_1 is an acyl radical of a glutaric acid.

24. The process of claim 22, wherein R_1 is an acyl radical of a adipic acid

25. The process of claim 22, wherein R_1 is an acyl radical of a subaric acid.

26. The process of claim 22, wherein R_1 is an acyl radical of a succinic acid.

27. The process of claim 22, wherein R_1 is an acyl radical of a sebacic acid.

28. The process of claim 16, wherein R_2 is a benzyl group.

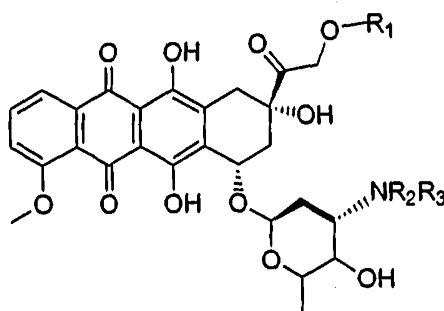
29. The process of claim 28, wherein R_1 is an acyl radical of an acid selected from the group consisting of an alkanoate having 4 to 17 carbon atoms, glutaric acid, adipic acid, subaric acid, succinic acid, acetic acid, octanoic acid, benzoic acid, propionic acid, phenylacetic acid, nicotinic acid, formic acid, butyric acid, glycolic acid, glycinic acid, succinic acid, 2'-hydroxy-naphthoic acid, cyclopentylpropionic acid, 2'-pyrrolcarboxylic acid, carbamic acid and ethylcarbonic acid.

30. The process of claim 29, wherein R_1 is an acyl radical of a valerate.

31. The process of claim 29, wherein R_1 is an acyl radical of a glutaric acid.

32. The process of claim 16 further comprising:

reacting the second product compound with a chemical alkylating agent under conditions effective to produce a third product compound of the formula:



where R_3 contains a substituted or unsubstituted C_1-C_{10} alkyl or benzyl group.

33. The process of claim 32, wherein R_1 is an acyl radical of an acid selected from the group consisting of acetic, octanoic, benzoic, propionic, phenylacetic, nicotinic, formic, butyric, glycolic, glycinic, succinic, 2'-hydroxy-naphthoic, cyclopentylpropionic, 2'-pyrrolcarboxylic, carbamic and ethylcarbonic acids and R_2 is $COCF_3$.

34. The process of claim 32, wherein R_1 is an acyl radical of an alkanoate having 4 to 10 carbon atoms and R_2 is $COCF_3$.

35. The process of claim 34, wherein the alkanoate is selected from the group consisting of valerate, butanoate, isobutyrate, hexanoate, and octanoate.

36. The process of claim 35, wherein the alkanoate is valerate.

37. The process of claim 32, wherein R_1 is an acyl radical of a dicarboxylic acid and R_2 is $COCF_3$.

38. The process of claim 37, wherein the dicarboxylic acid is selected from the group consisting of glutaric acid, adipic acid, subaric acid, and succinic acid.

39. The process of claim 38, wherein R_1 is an acyl radical of a glutaric acid.

40. The process of claim 38, wherein R_1 is an acyl radical of a adipic acid.

41. The process of claim 38, wherein R_1 is an acyl radical of a subaric acid.

42. The process of claim 38, wherein R_1 is an acyl radical of a succinic acid.

43. The process of claim 32, wherein R_2 is a benzyl group.

44. The process of claim 43, wherein R_1 is an acyl radical of an acid selected from the group consisting of an alkanoate having 4 to 17 carbon atoms, glutaric acid, adipic acid, subaric acid, succinic acid, acetic acid, octanoic acid, benzoic acid, propionic acid, phenylacetic acid, nicotinic acid, formic acid, butyric acid, glycolic acid, glycinic acid, succinic acid, 2'-hydroxy-naphthoic acid, cyclopentylpropionic acid, 2'-pyrrolcarboxylic acid, carbamic acid and ethylcarbonic acid.

45. The process of claim 44, wherein R_1 is valerate.

46. The process of claim 44, wherein R_1 is glutaric acid.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/41604

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07D 315/00; C12P 7/40, 7/24, 7/26

US CL : 549/417; 435/136, 147, 148

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)

U.S. : 549/417; 435/136, 147, 148

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHAIRES et al. Parsing the Free Energy of Anthracycline Antibiotic Binding to DNA. Biochemistry. 20 February 1996, Vol. 35, No. 7, pages 2047-2053.	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

18 March 2003 (18.03.2003)

Date of mailing of the international search report

01 APR 2003

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