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Nonaqueous Biocatalytic Synthesis of New Cytotoxic Doxorubicin Derivatives: Exploiting Unexpected Differences in the Regioselectivity of Salt-Activated and Solubilized Subtilisin

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Abstract: Two enzymes, *Mucor javanicus* lipase and subtilisin Carlsberg (SC), catalyzed the nonaqueous acylation of doxorubicin (DOX). Compared to the untreated enzyme the rate of DOX acylation at the C-14 position with vinyl butyrate in toluene was 25-fold higher by lipase ion-paired with Aerosol OT (AOT) and 5-fold higher by lipase activated by 98% (w/w) KCl co-lyophilization (3.21 and 0.67 μmol/min g-lipase, respectively, vs 0.13 μmol/min g-lipase). Particulate subtilisin Carlsberg (SC) was nearly incapable of DOX acylation, but ion-paired SC (AOT-SC) catalyzed acylation at a rate of 2.85 μmol/min g-protease. The *M. javanicus* formulations, AOT-SC, and SC exclusively acylated the C14 primary hydroxyl group of DOX. Co-lyophilization of SC with 98% (w/w) KCl expanded the enzyme's regiospecificity such that KCl-SC additionally acylated the C4′ hydroxyl and C3′ amine groups. The total rate of DOX conversion with KCl-SC was 56.7 μmol/min g-protease. The altered specificity of KCl-SC is a new property of the enzyme imparted by the salt activation, and represents the first report of unnatural regioselectivity exhibited by a salt-activated enzyme. Using AOT-SC catalysis, four unique selectively acylated DOX analogues were generated, and KCl-SC was used to prepare DOX derivatives acylated at the alternative sites. Cytotoxicities of select derivatives were evaluated against the MCF7 breast cancer cell line (DOX $IC_{50} = 27$ nM) and its multidrug-resistant sub-line, MCF7-ADR (DOX $IC_{50} = 27$ μM). The novel derivative 14-(2-thiophene acetate) DOX was relatively potent against both cell lines (IC_{50} of 65 nM and 8 μM, respectively) and the 14-(benzyl carbonate) DOX analogue was as potent as DOX against the MCF7 line (25 nM). Activated biocatalysts and their novel regioselectivity differences thus enabled single-step reaction pathways to an effective collection of doxorubicin analogues.

Introduction

Nonaqueous enzymology is an increasingly valuable tool for synthetic chemistry.[1] Within the unnatural environment of organic solvents, enzymes catalyze regioselective and enantioselective reactions under mild conditions with a broad range of substrates.[2] 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 with traditional synthetic methods. Recent advances in nonaqueous biocatalysis, including salt activation[3,4] and ion-paired solubilization,[5,6] inspired us to pursue a biocatalytic route toward novel analogues of doxorubicin (**1**, DOX), one of the most potent and extensively used chemotherapeutics in cancer treatment.[7]

Over thirty years ago, the anthracycline doxorubicin was first isolated from *Streptomyces peucetius* var. *Caesius* and recognized for its antitumor properties,[8] While DOX has remained one of the most successful chemotherapy agents on the market,[7,9] it has a number of therapeutic drawbacks including dose-limiting cardiotoxicity and susceptibility to multidrug resistance.[7,10,11] In the intervening decades thousands of DOX analogues have been developed in the largely unsuccessful search for an

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improved pharmaceutical.[11] As with many other natural compounds, the chemical synthesis of DOX is a challenging process. Several extensive procedures exist for generating DOX but none is convenient for rapid generation of diverse analogues.[12] Therefore, most efforts to modify DOX 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.[13]

The chemical fragility and functional diversity of DOX provide an excellent opportunity to take advantage of biocatalysis. Many of the original, and ongoing, efforts to develop anthracycline analogues have utilized microbial transformations.[14] In considering cell-free biocatalysis, commercially available hydrolases, glycosidases, peroxidases, and dehydrogenases could potentially be applied to generate, respectively, acylated, glycosylated, and oxidized or reduced derivatives. The abundant literature on DOX structure−activity relationships[15−17] provides some guidance for focusing analogue 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 DOX is intercalated, the C9 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. To date, promising doxorubicin derivatives predominately involve alterations to the sugar structure,[15,17,18] such as the attachment of alkylating agents, but a relatively small number of studies advocate the C9 site as a promising target for activity-enhancing modifications.[19−21] Both of these types of modifications were thus considered in the present work. Targeting the less derivatized part of the DOX molecule, emphasis was placed on enzymatic modification of the C14 primary hydroxyl while the two nucleophilic sites on the glycone were selected as additional targets.

Hydrolytic enzymes are commonly 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.[22] Accordingly, the regioselective acylation of DOX with salt-activated and solubilized proteases in a nonaqueous environment represents a unique pathway to achieve potent cytotoxic DOX analogues, and expands the repertoire of biocatalytic techniques, particularly those involving activated enzyme formulations, available for a combinatorial lead-development program. We report here the synthesis of unique DOX analogues with enzymatic catalysis. Moreover, we have discovered that the observed enzymic regioselectivity, and hence ultimate product diversity, can be manipulated by formulating the biocatalyst under different conditions.

Materials and Methods

Materials. Subtilisin Carlsberg (EC 3.4.21.14; alkaline protease from *Bacillus licheniformis*; 7−15 units/mg), Aerosol OT (AOT, dioctylsodium sulfosuccinate), Doxorubicin hydrochloride (DOX·HCl), buffers, salts, Dubelco's Modified Eagle's Medium (DMEM), dimethyl sulfoxide (DMSO), glutaraldehyde, crystal violet, trypsin, fetal bovine serum, and cell culture additives were purchased from Sigma Chemical Co. (St. Louis, MO) in the highest available grade or cell culture grade as needed. *Mucor javanicus* lipase (EC 3.1.1.3; 300 units/mg) was purchased from Fluka (Milwaukee, WI). T-150 and T-75 culture flasks were from Corning Inc. (Corning, NY). The silanized Hewlett-Packard HPLC vials used as reaction vessels and for sampling, acetic acid, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Triethylamine (TEA), chloroform, acetonitrile, toluene, and isooctane were from Aldrich (Milwaukee, WI) at HPLC grade or the highest available purity. Solvents used for reactions were dried over 3 Å molecular sieves (Linde) at least 24 h prior to use or before setting specific levels of hydration. Vinyl esters other than vinyl butyrate were purchased from the Biocatalysis Division of Albany Molecular Research, Inc. (Iowa City, IA).

Doxorubicin Preparation. Doxorubicin hydrochloride (DOX·HCl) has minimal solubility in organic solvents. Prior to nonaqueous reactions, the commercially obtained DOX·HCl required desalting. DOX·HCl was first dissolved into distilled and deionized water at ~1 mg/mL to form an orange-red solution. An equal volume of HPLC-grade chloroform was then contacted with the aqueous solution. Slow addition of triethylamine in slight molar excess resulted in a violet-tinted aqueous phase in which doxorubicin is unstable[13] and salt-free. Agitation rapidly transferred most of the DOX into the chloroform phase where it appeared deep orange. The chloroform layer was collected and the process of titration and extraction repeated until the aqueous phase was essentially colorless. Centrifugation was used to separate and remove bulk water from the combined chloroform extracts; additional dehydration was performed over sodium sulfate. The solution was then removed from the salt and sparged with dry nitrogen gas to rapidly evaporate the chloroform and yield dry orange-red solids. The resulting solids could be dissolved into many organic solvents, especially aromatic, halogenated, and polar liquids. Prior to dissolution, the solids were stored desiccated under reduced pressure at 4 °C.

Biocatalyst Screening. A total of 27 commercially available lipase and six protease preparations were screened for the ability to acylate

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doxorubicin. Vinyl butyrate was employed as the acyl donor in the screen. Reactions were performed with slurries of particulate enzyme mixtures, with each mixture containing three of the candidate biocatalysts. Each reaction was tested in three solvents (chloroform, toluene, and acetonitrile). Analytical TLC provided rapid assessment of reaction progress. HPLC analysis confirmed the presence of product in two enzyme mixtures. Subsequent tests with isolated enzymes identified *M. javanicus* lipase as the untreated enzyme that best acylated DOX. A second round of screening was then performed with salt-activated *M. javanicus* lipase, AOT ion-paired lipase, several additional salt-activated proteases, and AOT ion-paired subtilisin Carlsberg.

TLC Analysis in the Preliminary Biocatalyst Screen. Thin-layer chromatography (TLC) was used to reveal the production of DOX butyl amides and DOX butyl esters in initial biocatalyst screens. Polygram SIL G/UV_{254} precoated 0.25 mm silica gel sheets were spotted with reaction supernatant, and run with a mobile phase consisting of 3:1:1 (v:v:v) *n*-butanol:acetic acid:water. (An improved method was later developed that used 4:1 (v:v) chloroform:methanol as the mobile phase.) Unmodified doxorubicin and its C14 butyl ester derivative had R_f values of 0.45 and 0.59, respectively.

Liquid Chromatography and LC/MS Analysis. Doxorubicin reactions were analyzed using high performance liquid chromatography (HPLC) and mass spectrometry (MS). Derivatives were baseline-resolved in reverse-phase chromatography on a C-18 column (YMC ODS-A, 25 cm × 4.6 mm × 5 μm, 120 Å pore) with a 1 mL/min flow of an acetonitrile/buffer (pH 4.3, 40 mM acetate, 16 mM ammonia) gradient (35% acetonitrile 0−3 min, linear gradient to 98% at 18−23 min, to 33% at 24 min, to 35% from 25 to 30 min). The Hewlett-Packard (Agilent) 1100 LC/MSD was equipped with a diode array detector (DAD) and an atmospheric pressure electrospray ionization (API-ES) unit. Absorbance at 488 nm (4 nm bandwidth, 590 nm reference, 20 nm bandwidth) provided quantitative detection. Both positive- and negative-ion electrospray MS were used to confirm the molecular weight reaction products. Compounds tested for cytotoxic efficacy were purified on the same HPLC with use of a semipreparative 9.4 × 250 mm 5 μm ZORBAX SB-C18 column (Hewlett-Packard) and a Foxy 500 fraction collector. Proton NMR spectra were obtained on a 500 MHz Brukner instrument with samples dissolved in $CDCl_3$.

Enzyme Solubilization. Subtilisin Carlsberg protease was solubilized by ion pairing with AOT according to the procedure described for α-chymotrypsin by Paradkar and Dordick.[6] A 1 mg/mL aqueous solution of subtilisin Carlsberg was prepared in 10 mM bis-Tris propane buffer at pH 7.8 containing 2 mM $CaCl_2$. This solution was contacted with an isooctane phase containing 2 mM AOT with stirring at 24 °C for 20 min. The organic phase was transferred to centrifuge tubes and spun for 4 min at 3000 rpm to complete the removal of residual bulk water. The isooctane was dried off with N_2 sparging to yield an activated enzyme product that dissolved readily into most organic solvents. The lipase from *M. javanicus* was ion-paired in similar fashion by using a slightly different aqueous-phase buffer solution. For the lipase, the optimal aqueous solution contained 2 mg/mL lipase powder in a buffer of 10 mM bis-Tris propane, pH 6.0, and 300 mM NaCl. In the aqueous and organic phases the protein content was measured by absorbance at 280 nm, and concentrations were calculated by using experimentally determined extinction coefficients (1.22×10^3 au cm^2/g lipase and 732 au cm^2/g protease). Typical extraction efficiencies of 20−30% were observed for both enzymes.

KCl-Activation of Enzymes. Subtilisin Carlsberg protease and the *M. javanicus* lipase were prepared as 98%(wt) KCl catalysts with use of a previously developed method.[4] Solutions containing 98% (wt/wt) KCl, 1% enzyme powder (as supplied by the manufacturer), and 1% K_2PO_4 buffer were adjusted to pH 7.8, shell frozen with liquid nitrogen, and lyophilized for 69 h on a Labconco freeze-dryer.

Enzymatic Reactions. For the purpose of evaluating reaction rates, and for establishing reaction specificity, enzymatic DOX acylations were performed with 0.6 mL reactions within silanized glass HPLC vials. The standard protocol involved the dissolution of dry organic-solvent-soluble DOX into the reaction solvent at 1 mg/mL concentration with subsequent distribution of 0.3 mL into each reaction vial. Next, in the case of salt-activated enzymes, 6 mg of catalyst (0.06 mg enzyme) was added to the DOX solution, immediately followed by 0.3 mL of the acyl donor solution (25 μL of vinyl ester plus 275 μL of solvent). For AOT ion-paired enzymes, the acyl donor solution was used to redissolve the ion-paired enzyme to yield a 4 mg/mL enzyme solution and then 0.3 mL of the ester/enzyme solution was quickly transferred to each reaction vial of dissolved DOX. All reaction vials were sealed with Teflon-coated caps and then maintained at 30 °C with 250 rpm orbital shaking. The progress of all DOX experiments was monitored by periodic sampling (30 μL) and HPLC/MS analysis. Initial rates were determined from a least-squares fit of multiple data points in the first several hours of each assay, during which time the reaction trajectory remained linear.

Cell Culture. Two breast cancer cell lines were employed in cytotoxicity tests. The MCF7 cell line was obtained from Gary L. Firestone (U. C. Berkeley, Department of Molecular and Cellular Biology) at passage 22. A multidrug resistant cell line, originally derived from a DOX-challenged MCF7 culture and commonly referred to as MCF7-Adr, was purchased from the Frederick Cancer Research Facility DCT Tumor Repository at the National Cancer Institute. Cells were cultured as monolayers in T-150 and T-75 flasks using DMEM with L-glutamine, phenol red, and 4.5 g/L glucose with 1.2 g/L sodium bicarbonate and 3.6 g/L HEPES buffer adjusted to pH 7.1 and 10% (v/v) Fetal Bovine Serum. Cultures were incubated at 37 °C with a humidified 5% CO_2 atmosphere. Media for the multidrug resistant cells additionally contained 5 μM DOX·HCl to ensure maintenance of drug resistance. Cell lines were not used beyond their 44th passage.

Cytotoxicity Tests. A crystal violet staining procedure and 96-well plate cultures were used to assay cell viability and determine IC_{50} values. Well loading was accomplished by aliquoting 100 μL suspensions of 10,000 cells/mL (as measured with a hemocytometer) into the wells of 200 μL flat-bottomed 96-well culture plates. Once seeded, cells were cultured for 24 h in drug-free media. After 24 h the media was aspirated and replaced by media containing the appropriate concentration of DOX analogue. Each experimental compound was evaluated at 5−6 serially diluted concentrations. All tests were performed in triplicate and in parallel with vehicle (DMSO) and media controls. Challenged cultures were maintained without further media replacement for 4 days, at which time 15 μL of a 10% (v/v) glutaraldehyde solution was added to each well to halt the cultures. After 20 min, the glutaraldehyde-fixed cell plates were rinsed extensively in distilled water and dried overnight at reduced pressure. Before staining, each cell was pretreated in 100 μL of a 1% (v/v) acetic acid solution and dried again. Staining was accomplished by treating each well with 100 μL of a 0.1% (wt/v) crystal violet solution for 1 h at 24 °C. Excess stain was washed off under continuous flow of distilled water for 20 min and then the plates were dried. Acetic acid (100 μL, 10% (v/v)) was added to each well and, after 15 min on a plate agitator, the plates were scanned at 550 nm with a Bio-Tek Elx9000 multiwell plate reader. Cell viability was determined as a percentage of untreated cells and dose response curves were generated for each compound. IC_{50} values were set at the concentration that corresponded with 50% viability. The stability of DOX or the DOX derivatives was not monitored during the course of the cytotoxicity assays.

Results

Selection of a Biocatalyst System. The first screening effort identified *M. javanicus* lipase as the acylation catalyst giving the highest yield. MS and ^{1}H NMR confirmed that the exclusive product was the butyl ester of the C14 hydroxyl. Several unexpected results were observed in the second set of screening when the activated proteases, and the *M. javanicus* lipase, were

Scheme 1. The Set of Reaction Pathways Accessible to the Different Biocatalyst Preparations

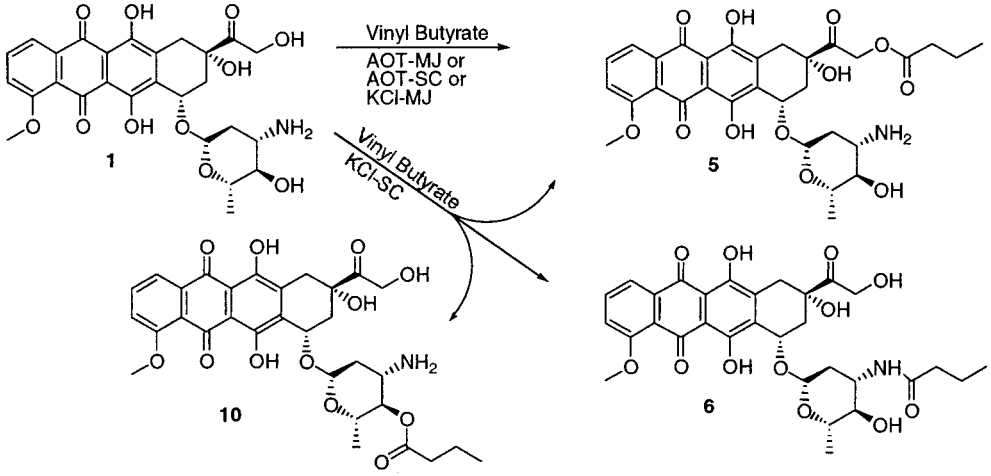


Table 1. Initial Rates[a] of Doxorubicin Acylation with Vinyl butyrate As Catalyzed by Several Enzyme Preparations

enzyme	activation method	initial rate (μmol/min g-protein)
M. javanicus lipase	AOT ion-pairing	3.21 ± 0.28
M. javanicus lipase	KCl co-lyophilization	0.67 ± 0.06
M. javanicus lipase	None	0.13 ± 0.02
subtilisin Carlsberg	AOT ion-pairing	2.85 ± 0.28
subtilisin Carlsberg	KCl co-lyophilization	56.7 ± 7.9

[a] Reactions in toluene at 37 °C. Initial rate for nonactivated subtilisin Carlsberg was too low to measure. For KCl-SC the rate is the summation of all doxorubicin acylations occurring simultaneously at three positions.

prepared by co-lyophilization with a 98-fold weight excess of KCl or by ion-paired solubilization. An impressive 25-fold enhanced rate (Table 1) was observed with the solubilized lipase (AOT-MJ). Surprisingly, despite showing negligible activity in its unmodified form, the acylation rates observed with the activated forms of subtilisin Carlsberg protease (SC) were similar to, or substantially exceeded, those of the activated lipase. Moreover, unique acyl derivatives were observed with KCl-activated SC preparation (KCl-SC), demonstrating a relaxed regioselectivity (Scheme 1). The significance of these results was 2-fold. First, the observation of high activity with KCl-SC and AOT-SC demonstrates the importance of performing enzyme screens with activated biocatalysts. Second, it was observed for the first time that two preparations of the same protease exhibited different regioselectivities under identical reaction conditions.

Reaction Development and Catalyst Selectivity. Biocatalyst performance in organic solvents is known to be highly sensitive to the reaction environment. Considerable enhancements can often be achieved by optimizing the critical parameters of solvent selection and solvent hydration. The initial rates of doxorubicin conversion provided measures of performance for salt-activated and solubilized forms of both subtilisin Carlsberg and *M. javanicus*. Toluene proved to be a significantly better solvent than chloroform, the solvent in which activity had originally been detected. With the exception of AOT-MJ, it was observed that in toluene the initial rate of conversion was greatest with water-saturated solvent. Salt-activated subtilisin was the best performing biocatalyst overall and the solubilized form of the enzyme was also highly activated (Table 1). The regioselectivity of AOT-SC was unaltered by changes in solvent hydration or the choice of solvent. Driving the reaction to completion with optimized conditions and extended times did not yield additional products with AOT-SC catalysis (Table 2). The dramatically altered nonregioselective behavior of KCl-SC was also constant across all the reaction environments tested.

The multisite activity of KCl-SC in DOX acylations was further investigated. Enzyme-free control reactions did not show any conversion, and the addition of KCl was also noncatalytic. Using conditions of higher enzyme concentration (60 vs 4 mg/mL) to overcome slow rates, the unmodified (salt-free) SC still exclusively produced the C14 ester product of DOX. These observations suggested that KCl activation had expanded the enzyme's regioselectivity beyond its natural range. Hydrolysis studies in aqueous media strengthened this hypothesis.

To observe the regioselectivity of the native enzyme during hydrolysis, mixtures of acylated DOX products and solutions of pure tributyl DOX were incubated with aqueous solutions of SC (10 mg/mL). Surprisingly, in both cases the resulting hydrolysis occurred exclusively at the C14 ester position. The additional acyl derivatives formed by KCl-SC are thus inaccessible substrates for the native enzyme. Finally, the addition of AOT to reactions catalyzed by KCl-SC did not restrict the site selectivity, although AOT did exert a nonsite-specific inhibitory effect when present in the reaction mixture at concentrations greater than 1 mM. KCl-SC can therefore be considered as a

Table 2. Regioselectivity Comparison of KCl-SC and AOT-SC Catalyzed Acylations of DOX with Vinyl Butyrate

		% molar distribution of acylated doxorubicin products[a]					
catalyst	DOX conv (%)	14-butyl ester, **5**	*N*-butyl amide, **6**	4′-butyl ester, **10**	*N*,14- dibutyrate, **7**	*N*,4′- dibutyrate, **8**	*N*,14,4′- tributyrate, **9**
KCl-SC	50	28	13	2	13	6	38
KCl-SC	95	12	19	10	32	12	15
AOT-SC	50	100					
AOT-SC	95	100					

[a] Values are averages of 3 independent experiments with product distributions calculated at 50% or 95% conversion of the starting doxorubicin material.

Chart 1. Doxorubicin: $R^1 = OH$, $R^2 = H$, $R^3 = H$.

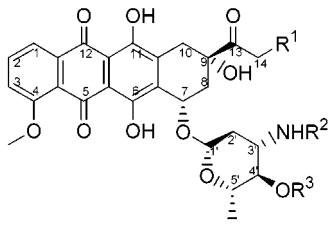
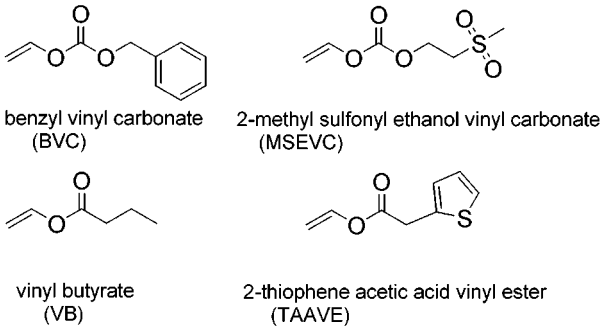


Chart 2. The Vinyl Ester Acyl Donors Used in the Enzymatic Synthesis of DOX Analogues



functionally distinct biocatalyst from either untreated or solubilized SC. The mechanistic basis for the functional difference between the two systems remains to be elucidated, as does the overall mechanism for the salt-activation effect in general.

Synthesis and Evaluation of Novel Doxorubicin Derivatives. Vinyl butyrate is an excellent substrate for protease-catalyzed transesterifications and is particularly effective for exploring reactions with new nucleophilic substrates. Having effected DOX transformations with vinyl butyrate, three additional compounds (Chart 2) were selected as promising acyl donors for derivatizing doxorubicin's primary hydroxyl group. The transformation of DOX with the vinyl esters and carbonates was accomplished by using the procedures developed with vinyl butyrate. Although the activity of AOT-SC toward the new donors varied by as much as 13-fold (data not shown), the selectivity of AOT-SC remained exclusive in catalyzing only primary hydroxyl acylations. Moreover, KCl-SC retained multisite acylation selectivity with DOX and the three additional donors.[23]

Since the amino group of DOX contributes significantly to DNA binding, derivatives blocking this site were of less interest. Nonetheless, following the theme of single-step biotransformations, four products (**6**−**9**, Table 3) that resulted from multisite KCl-SC acylation of DOX with vinyl butyrate were fully characterized in addition to the C14-doxorubicin butyrate product **5**. Cytotoxicity testing was exclusively performed on these five butyrate-modified DOX analogues and on the three analogues modified at the primary hydroxyl group. Nonhormone

Table 3. Doxorubicin and DOX Analogues Selected for Cytotoxicity Testing

compd	R^1	R^2	R^3	% yield[c]
1 (DOX)	OH	H	H	
2	2-thiophene acetate	H	H	94[a]
3	benzyl carbonate	H	H	51[a]
4	2-methyl sulfonyl ethyl carbonate	H	H	52[a]
5	butyrate	H	H	98[a]
6	OH	butyrate	H	19[b]
7	butyrate	butyrate	H	10[b]
8	OH	butyrate	butyrate	12[b]
9	butyrate	butyrate	butyrate	15[b]

[a] Yields for **2**−**5** are reported for AOT-SC catalyzed acylations. [b] **6**−**9** were competitively formed during the KCl-SC catalyzed reaction. [c] Based on conversion of DOX to product determined by HPLC.

Table 4. Cytotoxicity Values for DOX and the DOX Analogues as Tested with the Breast Cancer Cell Lines

compd	MCF7 IC_{50} (nM)	MCF7-ADR IC_{50} (μM)	RF[a]
1	27 ± 8	27 ± 2	1000
2	65 ± 32	8.0 ± 0.5	123
3	25 ± 5	ND[b]	
4	68 ± 4	111 ± 16	1630
5	164 ± 14	35 ± 8	213
6	365 ± 12	ND	
7	69 ± 3	101 ± 15	1464
8	1470 ± 480	ND	
9	389 ± 11	35 ± 3	90

[a] RF (resistance factor) = ratio of IC_{50} with resistant cells over IC_{50} for sensitive cells. [b] ND: the IC_{50} was not determined because it significantly exceeded the maximum tested concentration.

responsive breast cancers are among the most important clinical targets for DOX therapy. Therefore, two human breast cancer cell lines were employed for cytotoxicity tests. The MCF7 cell line is a well-established breast cancer line and MCF7-ADR is a DOX resistant variant. MCF7-ADR expresses several mechanisms of resistance including a common multidrug efflux pump.[24] Most DOX derivative studies result in analogues with reduced efficacy. The relative potency of DOX was indeed demonstrated in both cell lines as compared with the majority of new compounds (Table 4); however, even within the very modest library a few derivatives exhibited potency comparable to, or slightly greater than, DOX.

The cytotoxicity of the various butyl derivatives did not fall into a predictable hierarchy. In comparing the *N*,14-diacyl derivative **7** to the *N*- or 14-OH monoacyl compounds (**5** and **6**), or the triacyl analogue **9** to the *N*,4′-diacyl compound **8**, it is interesting to observe the increase in cytotoxicity against the MCF7 and MCF7-ADR cells when both the *N*- and 14-OH sites are modified. Also with the nonresistant line, the benzyl carbonate analogue had cytotoxicity equal to DOX and three other compounds exhibited slightly less than half the potency of DOX. For the multidrug-resistant cells, the 2-thiophene acetate ester product **2** was more than three times more potent than DOX and two of the butyl derivatives (**5** and **9**) showed toxicity comparable to that of DOX. The three derivatives with lower resistance factors (RF) than DOX were all modified at the C14−OH position. Modification at the primary hydroxyl did not ensure reduced RF values. While the relatively polar analogue **2** was particularly effective with the MDR line the

(23) The expanded regioselectivity of KCl-SC was tested only on an analytical scale with each of the additional donors, yielding multiple acylated products in the LC/MS chromatograms. In each case mass spectra of the products revealed the presence of at least one sugar-acylated derivative, one C14-acyl derivative, and one diacylated derivative of DOX in reactions with each donor. As a test of concept, a two-step strategy was employed wherein the four C14 modified derivatives, acylated by AOT-SC, were subsequently modified by KCl-SC using all possible paired combinations of the four acyl donors. The reactions were not scaled to provide structural data, but the UV/vis spectra of peaks in the LC chromatograms confirmed new DOX analogues in the product pools. At least 64 new analogues were generated out of the 124 (5^3 - 1) possible combinations of four acyl donors with DOX's three accessible nucleophilic groups.

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relative performance of the other three 14-O-esters did not correlate with the hydrophobicity of the acyl addition.

Discussion

For the first time, new derivatives of doxorubicin have been generated by applying nonaqueous enzymology. When solubilized and activated by AOT ion pairing, both subtilisin Carlsberg and *M. javanicus* lipase exclusively acylated DOX's primary hydroxyl moiety. This single-site reactivity matched the native regioselectivity of the nonactivated enzymes. The inability of native subtilisin Carlsberg to acylate the daunosamine sugar is consistent with a previous regioselectivity study investigating the enzyme's lack of reactivity with quercitrine, a flavanol with an identically configured sugar element.[25] In contrast, lyophilization of subtilisin Carlsberg as a 98% (w/w) KCl powder resulted in a catalyst with expanded reactivity. The ability of KCl-SC to acylate the two nucleophilic sites on the duanosamine sugar was an unexpected new property of the enzyme, more likely resulting from loss of positional sensitivity otherwise induced by the bulky aglycon than from a change in the active site's inherent stereo- or regioselectivity.

Ultimately, two preparations of the subtilisin protease provided radically different options for the development of new DOX analogues: highly or minimally selective single-step acylation. Both synthetic routes were implemented. Within the scope of the eight characterized analogues, primary hydroxyl esters resulting from selective DOX acylation had the greatest potency. The most active structures were the 2-thiophene acetate ester analogue **2,** nearly as cytotoxic as DOX against MCF7 cells and more potent with the resistant line, and the benzyl carbonate derivative **3**, having cytotoxic activity comparable to that of DOX against MCF7 cells. Neither derivative could be found in the previous literature, consistent with the relative absence of reports dealing with primary hydroxyl ester analogues.

Previously reported derivatives most similar to those developed here come from investigations into the effects of fatty acid chain length in 14-OH esters of DOX.[26] Within these studies the 14-O-butyrate ester was found to be too short to enhance MDR circumvention and the valerate derivative exhibited superior properties. Indeed, two valerate ester derivatives, *N*-trifluoroacetyladriamycin-14-valerate (AD 32) and *N*-benzyladriamycin-14-valerate (AD 198), have been extensively studied as prospective drugs with similar cytotoxicity to DOX and improved in vitro efficacy with multidrug resistant tumors.[19,20,26,27] The positive results with **2** and **3**, and the importance of similar derivatives AD 32 and AD 198, provide strong evidence that the types of structures readily accessible through biocatalysis can be effective chemotherapeutic candidates.

The unexpected patterns of efficacy among the variously butyrated DOX analogues further support the potential of biocatalysis for quickly generating libraries for structure−activity studies. Consider, for instance, the increase in cytotoxicity when the 14-OH was acylated in addition to the 3′-NH_2. Simultaneous acylation of DOX at two sites responsible for hydrogen bonding to DNA, and the presence of two aliphatic chains that might sterically hinder docking in the minor groove, would appear to lend to reduced effectiveness. The observation of the opposite result in both DOX-resistant and DOX-sensitive cells suggests that a primary contribution to the toxicity of such analogues involves a mechanism other than DNA intercalation.

Discoveries of two potent DOX derivatives, and the emerging patterns of structure−activity relationships in the cytotoxicity results within a very small collection of analogues, highlight the potential of combinatorial biocatalysis to yield valuable investigational drug libraries. Furthermore, the use of two forms of a single protease to achieve two different types of libraries, i.e., selective and nonselective acyl libraries, demonstrates the startling influence of enzyme activation on catalyst behavior while providing facile means for implementing complementary paths to the development of improved therapeutics.

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Supporting Information Available: Purification procedure and spectral data for previously undisclosed compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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