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Chemoenzymatic Synthesis of *N*-Trifluoroacetyl Doxorubicin-14-Valerate (Valrubicin)

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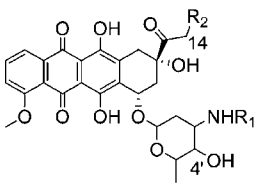
Abstract:

An efficient two-step, chemoenzymatic synthesis of *N*-trifluoroacetyl doxorubicin-14-valerate (Valrubicin) from doxorubicin hydrochloride salt is reported. The key step is a lipase-catalyzed regioselective esterification of *N*-trifluoroacetyl doxorubicin using commercially available valeric acid as the acyl donor. The overall yield for the process is 79%.



1. $R_1 = H$, $R_2 = OH$
2. $R_1 = COCF_3$, $R_2 = OCO(CH_2)_3CH_3$
3. $R_1 = R_2 = H$
4. $R_1 = COCF_3$, $R_2 = OH$
5. $R_1 = COCF_3$, $R_2 = H$

Figure 1. **Doxorubicin ($R_1 = H$ and $R_2 = OH$) and doxorubicin derivatives.**

1. Introduction

The anthracycline antibiotic doxorubicin **1** (Figure 1) has found significant value in the treatment of a variety of disseminated neoplastic conditions, including breast, ovarian, and lung carcinomas, acute lymphoblastic and acute myeloblastic leukemias, soft tissue and bone sarcomas, and Hodgkin's disease.[1] However **1** has a number of therapeutic drawbacks, including dose-dependent cardiotoxicity and acute myelosuppression.[2] The doxorubicin analogue *N*-trifluoroacetyl doxorubicin-14-valerate (**2**, Valrubicin) is of interest as it is known to exhibit significantly lower levels of cardiotoxicity in animals.[3,4] Valrubicin, an inhibitor of topoisomerase II and RNA synthesis,[5] is indicated in the treatment of refractory carcinoma in situ (CIS) of the bladder.[6]

The first practical synthetic route to **2** developed by Israel et al. entailed the *N*-trifluoroacetylation of daunorubicin **3** followed by C-14 iodination, and subsequent nucleophilic displacement with sodium valerate provided an overall yield for the process of ca. 24%.[7] Wang and co-workers have subsequently reported a similar, one-pot synthesis of **2**.[8] This process, however, also requires the chromatographic purification of **2** and affords **2** in only 35% yield. Also reported recently was a similar approach for the acylation of doxorubicin HCl that resulted in improved yields of the HCl salts of selected doxorubicin-14-*O*-esters.[9]

The utility of enzymes in achieving the selective modification of structurally complex substrates under mild conditions is well documented.[10] Since the amine moiety and C-4′ hydroxyl group of doxorubicin are known to be chemically more reactive than the C-14 hydroxyl group,[11] it has been necessary to protect the daunosamine moiety in order to regioselectively acylate the C-14 hydroxyl using traditional chemical approaches. On the other hand, two hydrolytic enzymes, *Mucor javanicus* lipase and substilisin Carlsberg ion-paired with Aerosol OT (which produces an organic solvent-soluble biocatalyst[12]), afforded the C-14 butyl ester of doxorubicin when vinyl butyrate was used as the acyl donor and toluene was the reaction solvent.[13] Optimization of this enzymatic system, however, did not provide a synthetic approach suitable for the preparative synthesis of gram quantities of doxorubicin-14-esters.[14] Therefore, we sought an efficient method for the synthesis of multigram quantities of **2** that utilizes an enzyme-catalyzed regioselective acylation of *N*-trifluoroacetyl doxorubicin **4**.

2. Results and Discussion

2.1. *N*-Trifluoroacetyl Doxorubicin Synthesis. The synthesis of **4** was carried out using the method developed for the production of *N*-trifluoroacetyl daunorubicin **5**

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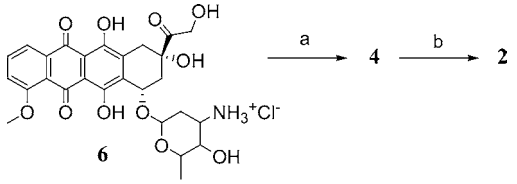
Scheme 1. **Chemoenzymatic synthesis of Valrubicin**[a]



[a] Reagents used: (a) $(CF_3CO)_2O$, pyridine −15 °C, quench H_2O; (b) Chirazyme L2,C3, valeric acid, MTBE/2-butanone (3:1 (v/v)).

(Scheme 1).[15] Pyridine was used as solvent for the reaction of doxorubicin with an excess of trifluoroacetic anhydride at −15 °C. Trifluoroacetylation of the C-14 and C-4′ hydroxyls is not problematic, as **4** is the sole product after quenching the reaction mixture with water and stirring for 20 min. The desired product **4** was obtained as a red solid in 88% yield after precipitation from 2-butanone/petroleum ether (bp 38−54 °C). NMR analysis of **4** was identical to that reported in the literature.[16]

2.2. Lipase-Catalyzed Regioselective Esterification Reaction. Doxorubicin contains one amine and four hydroxyl groups potentially amenable to enzymatic acylation (Figure 1). To identify biocatalysts capable of the regioselective derivatization of the C-14 hydroxyl of **4**, we tested over 80 commercially available lipases and proteases as acylation catalysts in a series of solvents using vinyl butyrate and valeric acid as model acyl donors.[17] Automation of the enzyme screen and mass spectrometric analysis[18] allowed for the rapid identification of Chirazyme L2,C3 (immobilized *Candida antarctica* lipase) as the best enzyme for the regioselective acylation of **4** with valeric acid (51% conversion of **4** to **2** in the screening assay).[17]

The employed heterogeneous enzyme catalyst suspended in the organic reaction mixture facilitates enzyme removal by simple filtration or centrifugation, as compared with the homogeneous catalyst,[13,14] and allows for the facile reuse of the catalyst. In addition, these enzyme 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. The heterogeneous enzyme catalyst also affords the possibility of a packed-bed reactor for the continuous production of **2**.

Subsequent research in our laboratory has shown that this regioselective acylation process is applicable to a wide variety of carboxylic acids (Scheme 1).[17] Indeed, the ability to use commercially available, inexpensive carboxylic acids, rather than their more expensive and less readily available activated ester counterparts, allowed for the synthesis of ca. 60 novel doxorubicin-14-esters for biological testing.[17]

Since water is generated in the esterification process, it is essential that water be removed in order to allow the reaction to proceed to completion. A variety of methods have been reported to facilitate water removal during lipase-catalyzed esterifications, including adding dehydrating agents (e.g., molecular sieves or sodium sulfate) directly to the reaction mixture,[19] microwave heating,[20] and azeotropic distillation.[21] We found that the most efficient method of water removal was to conduct the reaction in refluxing solvent using a flask fitted with a Dean−Stark and condenser assembly, and the solvent reservoir of the Dean−Stark apparatus was filled with 4 Å molecular sieves. Another approach that we examined (adding 4 Å molecular sieves directly to the reaction milieu) led to the loss of doxorubicin, presumably due to irreversible binding to the solid dehydrating agents. Indeed, the molecular sieves turned red during the course of the reaction (the reaction solvent turned clear), and repeated rinsing of the sieves with DMF did not recover significant quantities of doxorubicin and/or doxorubicin esters. Simply adding sodium sulfate to the reaction mixture also resulted in lower doxorubicin conversions than the Dean−Stark method under otherwise identical conditions (ca. 60% and 90% conversion, respectively).[17]

Not surprisingly, the choice of solvent for the lipase-catalyzed esterification has a substantial impact on process efficiency. The reaction solvent is also well-known to exert control over the regioselectivity of enzyme-catalyzed acylation reactions.[22] Although an exhaustive solvent survey was not performed, the selectivity of doxorubicin acylation is clearly dependent upon the reaction solvent (Table 1), and the appropriate solvent must be used in order to avoid overacylation at the C-4′ hydroxyl of **4**. Increased substrate solubility in the chosen reaction solvent will also enhance the reaction productivity, minimize effort needed in workup, and reduce material costs.

The reflux temperature of the solvent system is also key for enzyme selectivity, stability and reaction productivity. The temperature optimum of *Candida antartica* lipase is 60 °C in aqueous medium, and it is presumably advantageous for reuse of the enzyme to stay within this range, as significantly higher reaction temperatures may lead to enzyme inactivation.[23] While enzymes are substantially more thermostable in anhydrous organic solvents,[24] undesirable over acylation and/or side reactions can occur at higher reaction temperatures (Tables 1 and 2). It is further evident that the solvent should allow for efficient azeotropic removal of water and exhibit a low toxic potential, such as that found with class 3 solvents.[25]

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Table 1. **Esterification of 4 with valeric acid catalyzed by Chirazyme L2,C3 is affected by the reaction solvent[a]**

solvent	reflux temperature, °C	**4**, % remaining	conversion, %		
			to **2**	to 4′,14-*O*-diester	to other byproducts
MTBE	55	4	91	0	5
trifluoromethyl benzene	102	5	90	0	5
chloroform	61	38	60	0	2
MIBK	117	3	82	4	11
2-butanone	80	5	60	10	25

[a] reactions were performed as described for the synthesis of **2** (Experimental Section), with the exceptions of the concentration of **4** in the MTBE reaction (1 g/L) and the reflux temperature.

Table 2. **A binary mixture of MTBE and 2-butanone is an improved reaction solvent for the Chirazyme-catalyzed esterification of 4 with valeric acid**

MTBE:2-butanone	reflux temperature, °C	[**4**], g/L	reaction time, h	conversion of **4**, %	
				to **2**	to other byproducts
1:0	55	1	5	91	5
3:1	58	10	8	94	4
0:1	80	10	6	60	35

tert-Butyl methyl ether (MTBE) fulfilled all of the criteria for a suitable reaction solvent listed previously, apart from that of substrate solubility. The low solubility of **4** in MTBE (ca. 1 mg/mL) was not acceptable for the production of gram quantities of material. Although **2** is more soluble in MTBE (>20 mg/mL) than **4**, we favored a reaction system where **4** was soluble, thereby increasing the effective substrate concentration and minimizing handling issues with suspended doxorubicin. Indeed, **4** is well soluble in 2-butanone (>30 mg/mL), which is also a class 3 solvent. However, esterification of **4** with valeric acid in refluxing 2-butanone (bp 80 °C) using Chirazyme L2,C3 led to the formation of a significant amount (10%) of diacylated product along with several unknown side-products (25%) as seen by liquid chromatography/mass spectral (LC/MS) analysis (Table 1).[17] Other solvents examined (Table 1) were found to have an unfavorable boiling point for enzyme stability (trifluoromethyl benzene), poor enzyme activity (chloroform), or overacylation/byproduct formation (MIBK).

A suitable solvent system for the reaction was found to be a 3:1 (v/v) mixture of MTBE/2-butanone (Table 2). This binary solvent mixture allowed for an increased concentration of **4** (due to the high solubility of **4** in 2-butanone) at a lower refluxing temperature (MTBE and 2-butanone have bp of 55−56 °C and 80 °C, respectively). The substrate concentration was 10 g/L, and 10 equiv of valeric acid was used as the acyl donor.[26] Azeotropic distillation of the solvent mixture occurred at 58 °C. After 8 h, good conversion of **4** to the desired product **2** (94%) was observed by LC/MS analysis (Table 2). The enzyme was then removed by filtration and washed with 2-butanone (3 × 30 mL). The enzyme was air-dried and could be reused greater than five times without significant loss in activity.[27] The combined solvent was washed with saturated aqueous sodium hydrogen carbonate to remove excess valeric acid. After evaporation of the solvent under reduced pressure, precipitation from 2-butanone/petroleum ether (bp 38 to 54 °C) gave **2** as a red solid in 90% yield and >99% purity by LC/MS analysis. NMR analysis of **2** was identical to that reported in the literature.[8]

3. Summary

We have identified an efficient two-step, chemoenzymatic process to effect the synthesis of *N*-trifluoroacetyl doxorubicin-14-valerate **2** in an overall yield of 79%. The lipase-catalyzed esterification reaction is completely regioselective for the C-14-hydroxyl group of *N*-trifluoroacetyl doxorubicin **4**, and the enzyme can be reused without significant loss in activity (at least 5×). No chromatographic steps are required; consequently, the process is amenable to scale-up for the production of multigram quantities of material.

4. Experimental Section

General. All reagents and solvents used were obtained from Aldrich and used as received. Chirazyme L2,C3 was obtained from Biocatalytics, Inc. Doxorubicin HCl salt was obtained from Buckton Scott, Inc. LC/MS data were obtained using a PE Sciex API 100 LC/MS system. Proton NMR spectra were obtained on a Bruker Spectrospin spectrometer operating at 400 MHz.

***N*-Trifluoroacetyl Doxorubicin 4.** Doxorubicin HCl salt **6** (850 mg, 1.46 mmol) was stirred in dry pyridine (40 mL) under a nitrogen atmosphere at −15 °C for 15 min. Trifluoroacetic anhydride (13.14 mmol, 1.85 mL) in anhydrous diethyl ether (10 mL) was added dropwise over a 5-min period, and the reaction mixture was stirred at −15 °C for 3 h. Water (20 mL) was added, and stirring was continued for 20 min. The reaction mixture was extracted with ethyl acetate (2 × 50 mL), and the combined organic extracts were washed with water (3 × 20 mL) and saturated aqueous NaCl (20 mL) and then dried ($MgSO_4$) and evaporated under reduced

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(26) Given the relative cost of valeric acid and doxorubicin ($0.30/g and $90/g, respectively) on the research scale, a large (10-fold) molar excess of acid was employed in order to drive the esterification towards completion.

(27) Initial findings (>95% residual activity remaining after five reaction cycles without any enzyme regeneration attempted) indicate that the enzyme is quite stable in the identified reaction system, and additional enzyme recycling could further reduce catalyst costs.

pressure. Precipitation from 2-butanone/petroleum ether (bp 38−54 °C) gave the desired product (822 mg, 88%) as a red solid.

***N*-Trifluoroacetyl Doxorubicin-14-Valerate 2.** *N*-Trifluoroacetyl doxorubicin **4** (450 mg, 0.70 mmol) was dissolved in a MTBE/2-butanone mixture (3:1, 45 mL), and to this was added Chirazyme L2,C3 (2 g, 6290 units) and valeric acid (7 mmol, 0.76 mL). The reaction flask was fitted with a Dean−Stark and condenser assembly in which the receiver was filled with a mixture of 4 Å molecular sieves (ca. 15 g) and the reaction solvent. The reaction mixture was stirred vigorously and heated at reflux temperature (vapor temperature 58 °C) for 8 h. The enzyme was removed by filtration and washed with 2-butanone (3 × 30 mL). The solvent was washed with saturated aqueous sodium bicarbonate (4 × 30 mL) and saturated aqueous NaCl (30 mL) and then dried ($MgSO_4$) and evaporated under reduced pressure. Precipitation from 2-butanone/petroleum ether (bp 38−54 °C) gave *N*-trifluoroacetyl doxorubicin-14-valerate **2** (458 mg, 90%) as a red solid.

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