

Supporting information for

Chemo-enzymatic synthesis of valrubicin using Pluronic conjugated lipase with temperature responsiveness in organic media

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Experimental

Materials

Candida antarctic lipase B (CALB), Lipase from *Candida rugosa* (Type VII, L-1754) and Lipase from *Thermomyces lanuginosus* (LTL), *p*-nitrophenyl butyrate, Pluronic® F-127, Dess-martin periodinane, vinyl valerate were purchased from Sigma-Aldrich Co. LLC. Doxorubicin hydrochloride was purchased from Melone Pharmaceutical Co., Ltd, Dalian, China. Trifluoroacetic anhydride was purchased from Alfa Aesar, China.

Methods

Synthesis of enzyme-Pluronic nanoconjugates

The preparation of enzyme-Pluronic nanoconjugates was described by Zhu et al.¹ Pluronic-F127 was functionalized with terminal aldehyde groups by Dess-Martin periodinane. CALB, LTL, and CRL samples were pretreated by dialysis against boric acid buffer (10 mM, pH 8.0) prior to use. A solution of aldehyde-Pluronic (the ratio of aldehyde groups to amine groups of protein being as 1.1:1) was added to a protein solution with concentration of 5~10 mg/mL in borate buffer (50mM, pH=8.0). After 2 h reaction, NaCNBH₃ (10% weight of Pluronic) was added to the mixture for reducing the Schiff base for 4 h under room temperature. Then the mixture was dialyzed against the phosphate buffer (10 mM, pH 7.0) to remove small molecules (Mw <13,000).

Enzymatic activity assay

The hydrolytic activities of LTL, CRL, CALB, and their conjugates in aqueous solution were determined by using 4-nitrophenyl butyrate (p-NPB) as the substrate. Briefly, 3.8 mg of p-NPB was first dissolved in acetone and then diluted with 20 mL phosphate buffer (10 mM, pH 7.0) containing 1.25% (w/v) Triton X-100. The assay was started by adding 50 µL of enzyme solution (about 100 µg/mL) into 950 µL of substrate solution, and the increase of absorbance was detected at 348 nm (carried out on UV/Vis spectrophotometer, Shimadzu UV2550).

N-Trifluoroacetyl doxorubicin

The synthesis of the N-trifluoroacetyl doxorubicin was performed according to the method described by Mervyn Israel.² Briefly, 800 mg (1.3 mmol) of doxorubicin hydrochloride was dissolved in 8 mL of dry pyridine at -20 °C (ice-salt bath) for 15 min. Then, 2.8 mL of trifluoroacetic anhydride dissolved in 10 mL of anhydrous ester was added dropwise over a period of 10 min. After 20 min, 10 mL of water was added to quench the reaction. The mixture was extracted with ethyl acetate for 3 times. The extracts were merged, washed with deionized water,

and dried with Na₂SO₄. Then the excess ethyl acetate was evaporated. The crude product was obtained by precipitation from CHCl₃-petroleum ether, further purification was carried out by chromatography on a silica gel (elution with CHCl₃/CH₃OH).

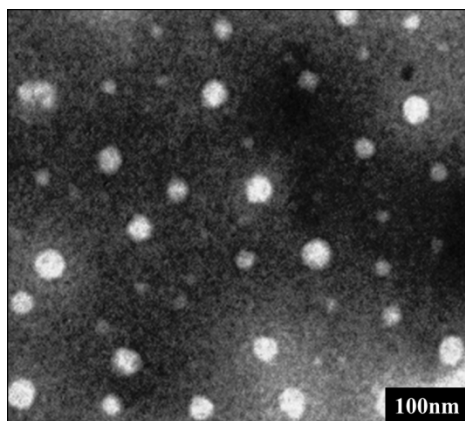
***N*-Trifluoroacetyl doxorubicin-14-valerate (valrubicin)**

The esterification of the *N*-trifluoroacetyl doxorubicin was catalyzed by lipase-Pluronic nanoconjugates using vinyl valerate as the acylation reagent. In a typical experiment, 100 mg of *N*-trifluoroacetyl doxorubicin (0.156 mmol) and 180 mg (1.4 mmol) vinyl valerate were dissolved in organic solvent, e.g. 10 mL of methyl isobutyl ketone, followed by the addition of 250 mg of CALB-Pluronic nanoconjugate (containing 20mg CALB). The mixture was stirred at 50 °C for 8h. The reaction was monitored by the thin-layer-chromatography (TLC, CH₃OH-CHCl₃, 1:10) and HPLC analysis. The solvent was evaporated and the crude product was precipitated from CHCl₃-petroleum ether and further purified by chromatography on a silica gel column (elution with CHCl₃/CH₃OH).

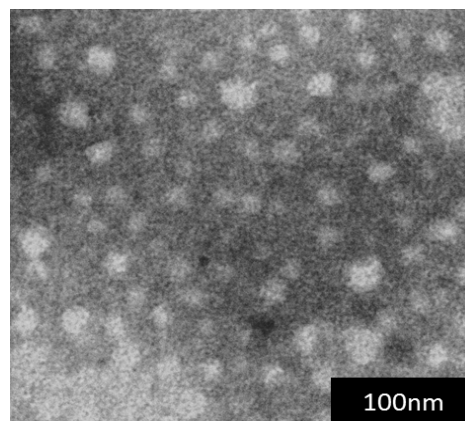
HPLC analysis

HPLC analysis was carried out on a SHIMADZU HPLC system by using a Kromasil C-18 column (250×4.6mm, 5μm). For the detection of *N*-trifluoroacetyl doxorubicin, the mobile phase was an isocratic mixture of MeOH-H₂O-H₃PO₄ (500:500:0.68, pH 2.6). For valrubicin, the mobile phase was an isocratic mixture of 75% acetonitrile and 25% 0.015 mol/L phosphoric acid solution. And the analyses were performed at 1 mL/min by UV/Vis absorbance at 254nm and 485nm.

TEM image of lipase conjugate in organic solvent



LTL- pluronic conjugate in MIBK



CRL-pluronic conjugate in MIBK

Fig. S1. LTL-, CRL-pluronic conjugates in MIBK.

HPLC of TF-Dox and valrubicin

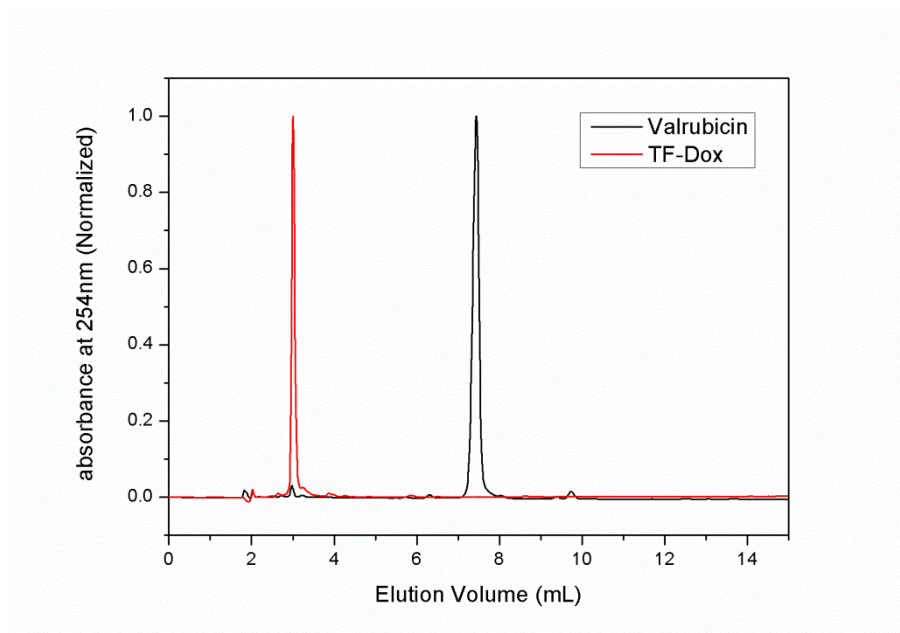


Fig. S2. HPLC of TF-Dox and valrubicin.

Mass spectrums of *N*-trifluoroacety doxorubicin and valrubicin

ESI-Q-TOF MS detections were carried out using the MicroTOF-Q (Bruker Daltonics Inc., USA) instrument.

N-Trifluoroacety Doxorubicin, $M=C_{29}H_{28}F_3NO_{12}$, $[M+Na]^+$: 662.1456

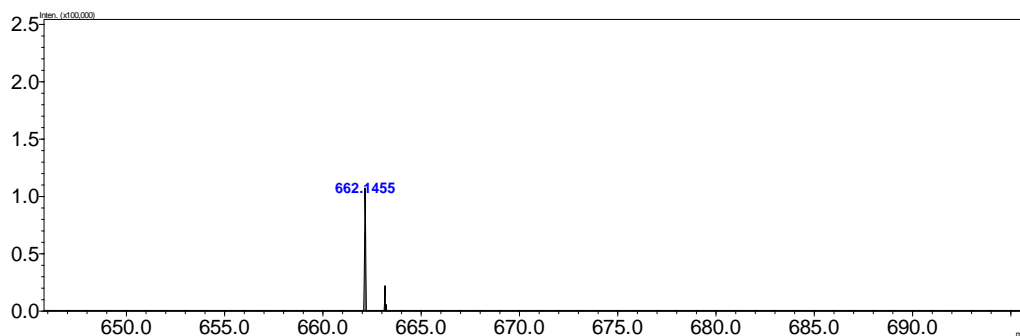


Fig. S3. Mass spectrum of TF-Dox.

Valrubicin, $M= C_{34}H_{36}F_3NO_{13}$, $[M+Na]^+$: 746.2031

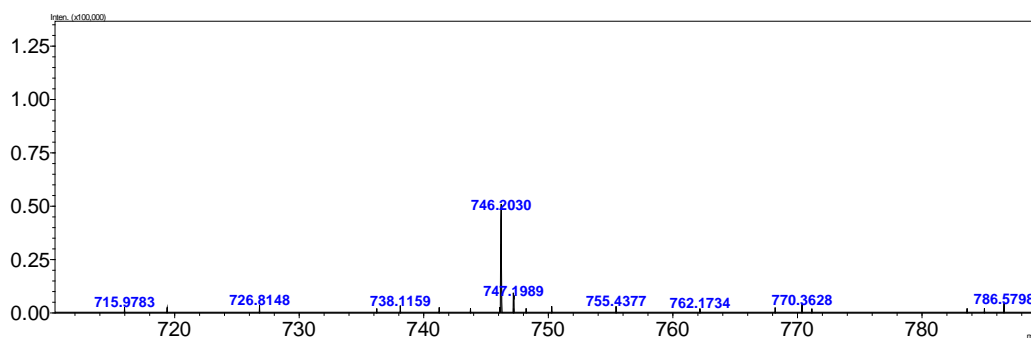
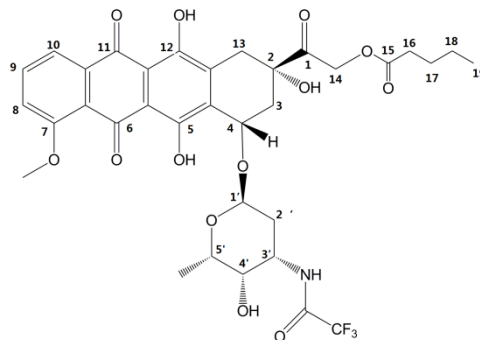


Fig. S4. Mass spectrum of valrubicin.

¹H-NMR of valrubicin and ¹³C-NMR spectrums of *N*-trifluoroacetyl doxorubicin and valrubicin

The NMR spectrum were carried out on a JEOL JNM-ECA600 spectrometer.



¹H-NMR of Valrubicin (600-MHz, Acetone-d₆): δ 7.85~7.86 (m, 2H), 7.58 (d, J=2Hz, 1H), 5.48 (s, 1H), 5.35 (d, J=18Hz, 1H), 5.17 (d, 2H), 4.36 (m, 1H), 4.23 (m, 1H), 4.02 (s, 3H), 3.72(s, 1H), 3.10 (d, J=18.5Hz, 1H, H-13e), 2.89 (d, J=18.5Hz, 1H, H-13a), 2.42 (t, J=7.8Hz, 2H), 2.10~2.15 (m, 2H), 1.60~1.80 (m, 2H, H-2'e and H-2'a), 1.40(m, 2H), 1.29 (d, J=6.5Hz, 3H), 1.25 (m, 2H), 0.90 (t, J=6Hz, 3H).

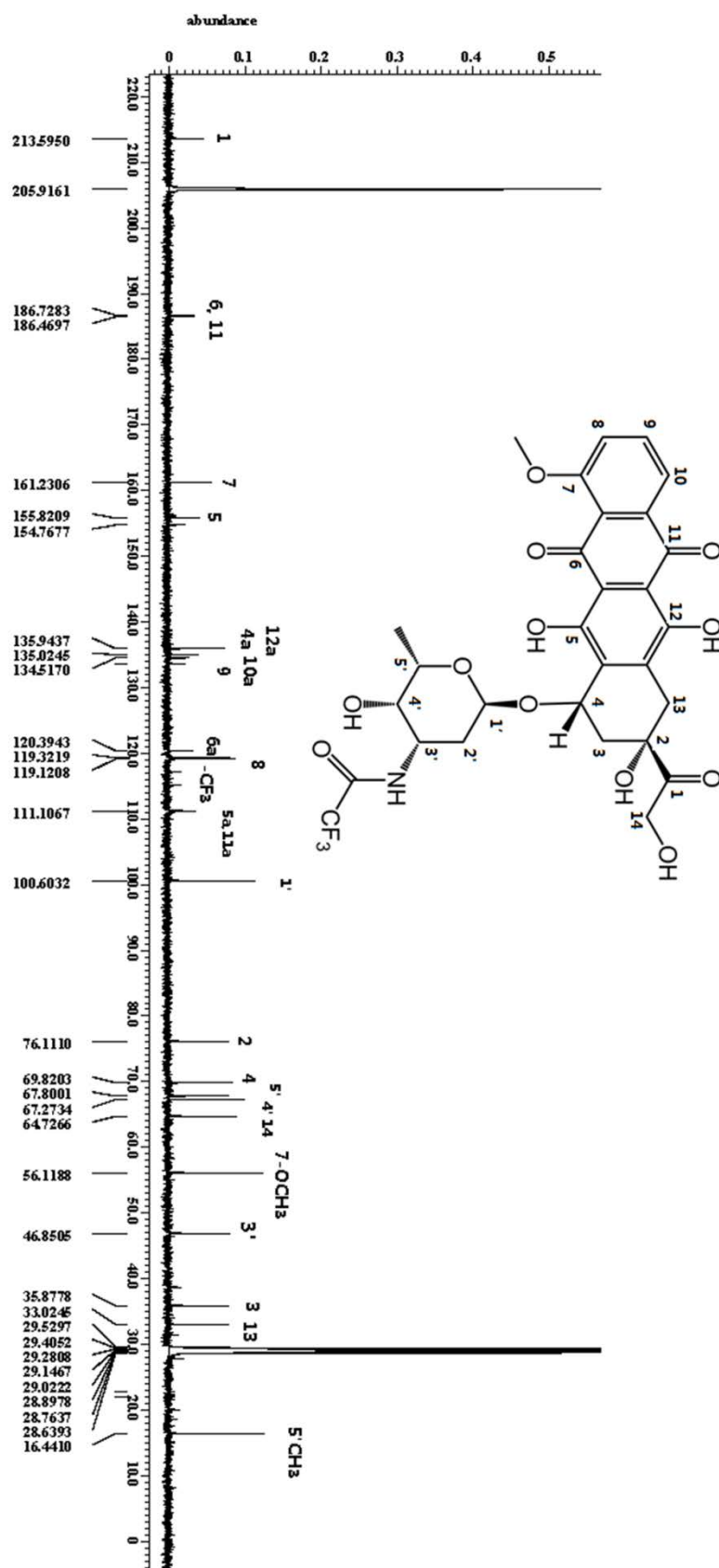


Fig. S5. ¹³C-NMR of TF-Dox.

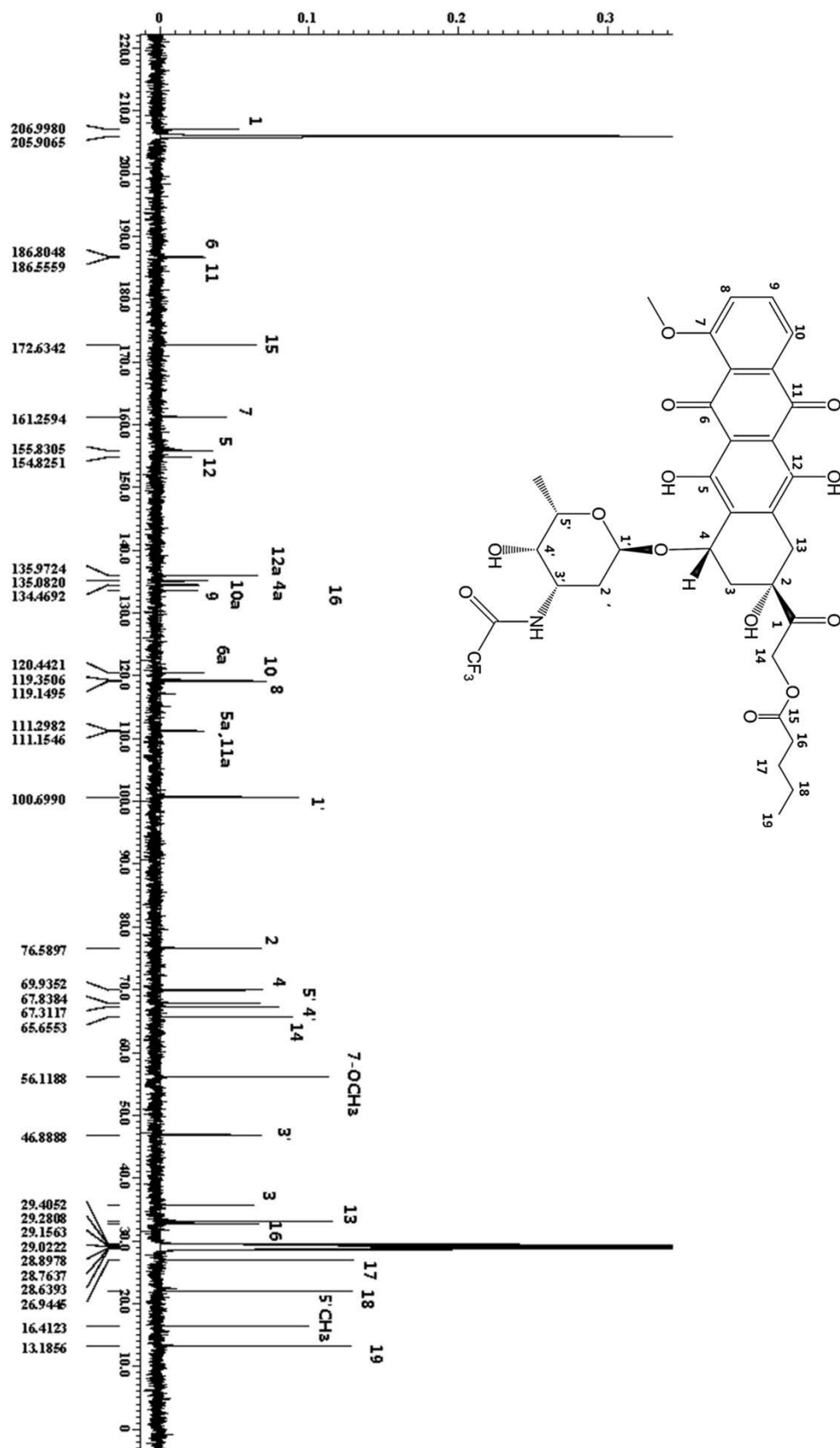


Fig. S6. ¹³C-NMR of valrubicin.

References:

1. J. Zhu, Y. Zhang, D. Lu, R. N. Zare, J. Ge and Z. Liu, *Chem. Commun.*, 2013, **49**, 6090-6092.
2. M. Israel, D. Taube, R. Seshadri and J. M. Idriss, *Journal of Medicinal Chemistry*, 1986, **29**, 1273-1276.