Electronic Supplementary Material (ESI) for Green Chemistry.

Electrical Supporting Information

Design of a self-sufficient hydride-shuttling cascade for concurrent bioproduction of 7,12-dioxolithocholate and L-*tert*-leucine

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Contents

1. Experimental Section

1.1 General information

Cholic acid (3α,7α,12α-trihydroxy-5β-cholanic acid; CA), deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid; DCA), and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanic acid; CDCA) were purchased from Aladdin Reagents Co., Ltd. (Shanghai, China). Trimethylpyruvic acid (TMP) and L-*tert*-leucine were obtained from Adamas Reagent Co., Ltd. (Shanghai, China). Standards of 7-oxo-deoxycholic acid (3α,12α-dihydroxy-7-oxo-5β-cholanic acid; 7-oxo-DCA), 12-oxo-chenodeoxycholic acid (3α,7α-dihydroxy-12-oxo-5β-cholanic acid; 12-oxo-CDCA), and 7,12-dioxo-lithocholic acid (3α-hydroxy-7,12-dioxo-5β-cholanoic acid; 7,12-dioxo-LCA) were enzymatically synthesized in our previous work.[S1] Unless otherwise specified, all other chemicals and reagents were obtained commercially and were of reagent grade. PrimeSTAR™ HS, Taq DNA polymerase, and restriction endonucleases were all purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The Gibson assembly kit was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). *E. coli* DH5α and *E. coli* BL21 (DE3) were used as hosts for gene cloning and protein expression, respectively. Plasmids pET-28a (+), pETDuet-1, and pRSFDuet-1 (Novagen, Shanghai, China) were used for the heterogeneous expression of recombinant protein. The recombinant plasmids pET28a (+)-*Ec*7α-HSDH, pET28a (+)-*Rr*12α-HSDH and pET28a (+)-*Es*LeuDH were previously constructed by our laboratory.[S1–S3] The molar yield of 7,12-dioxo-LCA were determined by HPLC analysis using a Shimadzu LC-2010A high performance liquid chromatography equipped with an Hypersil ODS2 column (250 mm × 4.6 mm, 5 μm particle size, Elite) and a UV detector. ^{1}H NMR and ^{13}C NMR were measured on a Bruker Ascend spectrometer.

1.2 Gene sequences

Gene sequence of *Rr*12α-HSDH (GenBank: QDC17258.1)

ATGAAACTGCGCGGGAAGACCGCCGTCGTCACCGGCGGTGCGGGCGGGATCGGCCGCGCGGTGACCCGCG
TGTTCGTCCGCGAGGGCGCCCGCGTGCTGTTCGTCGACGTCGACGACGATCGGGGGCGCGCGCTCGAGTCC
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AGATCCGCGACGCCGCCGTCGCGGCGTTCGGCGGCATCGACATCCTGGTCAACAACGCGCACGCGTCGCGC

CAGGCACTGCTGGTCGAGCACACCCCGGAGATGTTCGAGCTGTCGTTCGGCACGGGGTTCTACCCCACCGTG
CACCTCATGCAGGCCTGCTACCCGCAGCTCAAGCAGGCCCGGGGTTCCGTCGTCAACTTCGGCTCCGGGTCC
GCCCTCGACGGCATGCCGACGCAGACGTCGTACGCGGCGGCGAAGGAGGCGATCCGGGCGGTCAGCCGGG
TGGCCGCGAACGAATGGGCCGCCGACGGCATCCGCGTCAACGTCGTGTGCCCGTTCGCCGCGACCGAAGGC
GTGCAGGCCTGGCAGCAGGCGTTCCCCGACCGGGCGGCCGCCGCGGCGGCGAAGGTGCCGTTGCAGCGCA
TCGGCGACCCGGAGACGGACATCGCGCCGGTGGTGGTGTTCCTCGCCTCCGACGACTCGAAGTACATGACG
GGGCAGACGCTGATGGCCGACGGGGGCAGCATCAAGCTGCGGTGA

Gene sequence of *Ec*7α-HSDH (GenBank: BAA01384.1)

GTGTTTAATTCTGACAACCTGAGACTCGACGGAAAATGCGCCATCATCACAGGTGCGGGTGCAGGTATTGGT
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AACCATGTTGTAGACGAAATTCAACAACTGGGTGGTCAGGCATTTGCCTGCCGTTGTGATATTACTTCCGAAC
AGGAACTCTCTGCACTGGCAGACTTTGCTATCAGTAAGCTGGGTAAAGTTGATATTCTGGTTAACAACGCCG
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CTGGGCCAACCGCAAGATATTGCTAACGCAGCGCTGTTCCTTTGCTCGCCTGCTGCGAGCTGGGTAAGCGGA
CAAATTCTCACCGTCTCCGGTGGTGGGGTACAGGAGCTCAATTAA

Gene sequence of *Es*LeuDH (GenBank: ACB60396.1)

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TGAGCGGGCGATGAAAAAAGTCGAACTCGTCTATGATGCGGTAGCAAAAGTCATCGAAATTGCCAAACGTG
ACCATCTGCCGACTTACCGGGCAGCAGAGAAGATGGCAGAAGAACGGATCGCGACAATGGGCAGTGCCCG
CAGCCAGTTCTTACGCCGGGATAAAAACATTTTAGGATCACGCGGTTAA

1.3 Calculation of E factors

E factors were calculated as $Weight_{waste}/Weight_{product}$. The calculations did not take into account the waste generated for the preparation of the catalysts.

(1) 12α-HSDH/7α-HSDH/LeuDH system, 100 mM CA, 250 mM TMP, 50 mL (this work).

	Compound	Weight (mg)	Quantity (mg per mg products)
Product	7,12-dioxo-LCA + L-*tert*-leucine	1600 + 850	
Waste	CA	400	0.16
	Trimethylpyruvic acid	750	0.31
	Cells	1000	0.41
	Ammonium chloride	2650	1.08
	H_2O	50000	20.4
	1 M HCl	1200	0.49
	Ethanol	39500	16.1
E factor	Including water		38.9
	Excluding water		18.5

(2) O_2/NOX system, 10 mM CA, 75 mL, flow reactor (ref. 43).

Compound	Weight	Quantity

		(mg)	(mg per mg product)
Product	12-oxo-CDCA	297	
Waste	CA	3	0.01
	NAD^+	35.5	0.12
	12α-HSDH	0.26	0.001
	NOX	37.5	0.13
	MeOH	5925	19.9
	50 mM KPi pH 8.0	640	2.15
	H_2O	67500	227.3
E factor[a]	Including water		249.6
	Excluding water		22.3

[a] The calculations did not take into account the downstream processes.

(3) Syringaldazine/MtLac system, 120 mM CA, 410 mL (ref. 44).

	Compound	Weight (mg)	Quantity (mg per mg product)
Product	12-oxo-CDCA	18400	
Waste	CA	1600	0.09
	NAD^+	136	0.007
	Syringaldazine	21	0.001
	12α-HSDH	5.33	0.0003
	*Mt*Lac	139	0.008
	DMSO	45100	2.45
	100 mM KPi pH 7.2	6700	0.36
	H_2O	369000	20.1
	Diethyl ether	710000	38.9
E factor	Including water		61.6
	Excluding water		41.5

(4) Pyruvate/LDH system, 200 mM CA, 100 mL (ref. 36).

	Compound	Weight (mg)	Quantity (mg per mg product)
Product	12-oxo-CDCA	6800	
Waste	CA	1200	0.18
	Pyruvate	2640	0.39
	NAD^+	35.5	0.005
	Cells	1000	0.15
	100 mM KPi pH 8.0	1720	0.25
	H_2O	100000	14.7
	1 M HCl	4800	0.71
	Ethanol	79000	11.6
E factor	Including water		28.0
	Excluding water		13.3

(5) Acetone/Alcohol Dehydrogenase (ADH) system, 100 mM CA, 10 mL (ref. 42).

	Compound	Weight (mg)	Quantity (mg per mg product)
Product	12-oxo-CDCA	170	
Waste	CA	230	1.35
	Acetone	1970	11.6
	$NADP^+$	0.75	0.004
	12α-HSDH	16.7	0.1
	Dithiothreitol	1.6	0.01
	Bovine serumalbumin	60	0.35
	100 mM KPi pH 8.0	172	1.01
	H_2O	10000	58.8
	Ethyl acetate	9000	52.9

E factor	Including water	126.2
	Excluding water	67.4

(6) α-Ketoglutarate/Glutamate Dehydrogenase (GluDH) system, 100 mM CA, 70 mL (ref. 39).

	Compound	Weight (mg)	Quantity (mg per mg products)
Product	12-oxo-CDCA	2464	
Waste	CA	336	0.14
	a-Ketoglutarate	1691	0.69
	$NADP^+$	3.5	0.001
	12α-HSDH	10.8	0.004
	GLuDH	0.58	0.0002
	Dithiothreitol	11	0.004
	Sodium azide	14	0.006
	Ammonium acetate	1348	0.55
	100 mM KPi pH 8.0	1207	0.49
	H_2O	70000	28.4
	Acetone	276500	112.2
	Ethanolamine	427	0.17
E factor	Including water		142.7
	Excluding water		114.3

2. Supporting Tables

Table S1. Primers used for constructing recombinant *E. coli* strains.

Primer	Sequence (5'-3')
mcs1-12α-FP (BamHⅠ)	TCATCACCACAGCCAGGATCCGATGAAACTGCGCGGGAAGA
mcs1-12α-RP (HindⅢ)	TTAAGCATTATGCGGCCGCAAGCTTTCACCGCAGCTTGATGCTGC
mcs1-7α-FP (BamHⅠ)	TCATCACCACAGCCAGGATCCGATGTTTAATTCTGACAACC
mcs1-7α-RP (HindⅢ)	TTAAGCATTATGCGGCCGCAAGCTTTTAATTGAGCTCCTGTAC
mcs1-Es-FP (BamHⅠ)	TCATCACCACAGCCAGGATCCGATGGTTGAAACAAACG
Mcs1-Es-RP (HindⅢ)	TTAAGCATTATGCGGCCGCAAGCTTTTAACCGCGTGATCCT
mcs2-12α-FP (NdeⅠ)	GTATAAGAAGGAGATATACATATGATGAAACTGCGCGGGAAGACCGC
mcs2-12α-RP (KpnⅠ)	TTTCTTTACCAGACTCGAGGGTACCTCACCGCAGCTTGATGCT
mcs2-7α-FP (NdeⅠ)	GTATAAGAAGGAGATATACATATGATGTTTAATTCTGACAACCTGAGACTCG
mcs2-7α-RP (KpnⅠ)	TTTCTTTACCAGACTCGAGGGTACCTTAATTGAGCTCCTGTA
mcs2-Es-FP(NdeⅠ)	GTATAAGAAGGAGATATACATATGATGGTTGAAACAAACGTAGAAGCACG
mcs2-RP(KpnⅠ)	TTTCTTTACCAGACTCGAGGGTACCTTAACCGCGTGATCCTAAAATGTT
pETDuet-MCS1-FP	ATCGGTGATGTCGGCGATAT
pRSFDuet-MCS1-FP	GTTTTGCGCCATTCGATGGT
Duet-MCS1-RP	GATTATGCGGCCGTGTACAA
Duet-MCS2-FP	TTGTACACGGCCGCATAATC
Duet-MCS2-RP	TGCTAGTTATTGCTCAGCGG

Table S2. Strains and plasmids used in this study.

Strain and plasmids	Relevant characteristics	Source
pRSFDuet-1	double T7 promoters, RSF ori, Kan^R	Novagen
pETDuet-1	double T7 promoters, pBR322 ori, Amp^R	Novagen
pET28a (+)	T7 promoter, pBR322 ori, Kan^R	Novagen
pET28a (+)-*Rr*12α-HSDH	pET28a (+) carrying *12α-hsdh*	laboratory stock
pET28a (+)-*Ec*7α-HSDH	pET28a (+) carrying *7α-hsdh*	laboratory stock
pET28a (+)-*Es*LeuDH	pET28a (+) carrying *leudh*	laboratory stock
pRSFDuet-*Rr*12α-HSDH-*Es*LeuDH	pRSFDuet carrying *12α-hsdh* and *leudh*	this study
pRSFDuet-*Rr*12α-HSDH	pRSFDuet carrying *12α-hsdh*	this study
pRSFDuet-*Rr*12α-HSDH-*Ec*7α-HSDH	pRSFDuet carrying *12α-hsdh* and *7α-hsdh*	this study
pRSFDuet-*Es*LeuDH-*Rr*12α-HSDH	pRSFDuet carrying *leudh* and *12α-hsdh*	this study
pRSFDuet-*Es*LeuDH	pRSFDuet carrying *leudh*	this study
pRSFDuet-*Es*LeuDH-*Ec*7α-HSDH	pRSFDuet carrying *leudh* and *7α-hsdh*	this study
pETDuet-*Ec*7α-HSDH	pETDuet carrying *7α-hsdh*	this study
pETDuet-*Ec*7α-HSDH-*Es*LeuDH	pETDuet carrying *7α-hsdh* and *leudh*	this study
pETDuet-*Rr*12α-HSDH	pETDuet carrying *12α-hsdh*	this study
pETDuet-*Rr*12α-HSDH-*Ec*7α-HSDH	pETDuet carrying *12α-hsdh* and *7α-hsdh*	this study
pETDuet-*Es*LeuDH	pETDuet carrying *leudh*	this study
Strain 1	*E. coli* BL21(DE3)/(pRSFDuet-*Rr*12α-HSDH-*Es*LeuDH and pETDuet-*Ec*7α-HSDH-*Es*LeuDH)	this study
Strain 2	*E. coli* BL21(DE3)/(pRSFDuet-*Rr*12α-HSDH and pETDuet-*Ec*7α-HSDH-*Es*LeuDH)	this study

Strain 3	*E. coli* BL21(DE3)/(pRSFDuet-*Es*LeuDH-*Rr*12α-HSDH and pETDuet-*Ec*7α-HSDH-*Es*LeuDH)	this study
Strain 4	*E. coli* BL21(DE3)/(pRSFDuet-*Rr*12α-HSDH-*Es*LeuDH and pETDuet-*Ec*7α-HSDH)	this study
Strain 5	*E. coli* BL21(DE3)/(pRSFDuet-*Es*LeuDH-*Rr*12α-HSDH and pETDuet-*Ec*7α-HSDH)	this study
Strain 6	*E. coli* BL21(DE3)/(pRSFDuet-*Es*LeuDH and pETDuet-*Rr*12α-HSDH-*Ec*7α-HSDH)	this study
Strain 7	*E. coli* BL21(DE3)/(pRSFDuet-*Es*LeuDH-*Ec*7α-HSDH and pETDuet-*Rr*12α-HSDH)	this study
Strain 8	*E. coli* BL21(DE3)/(pRSFDuet-*Rr*12α-HSDH-*Ec*7α-HSDH and pETDuet-*Es*LeuDH)	this study

3. Supporting Figures

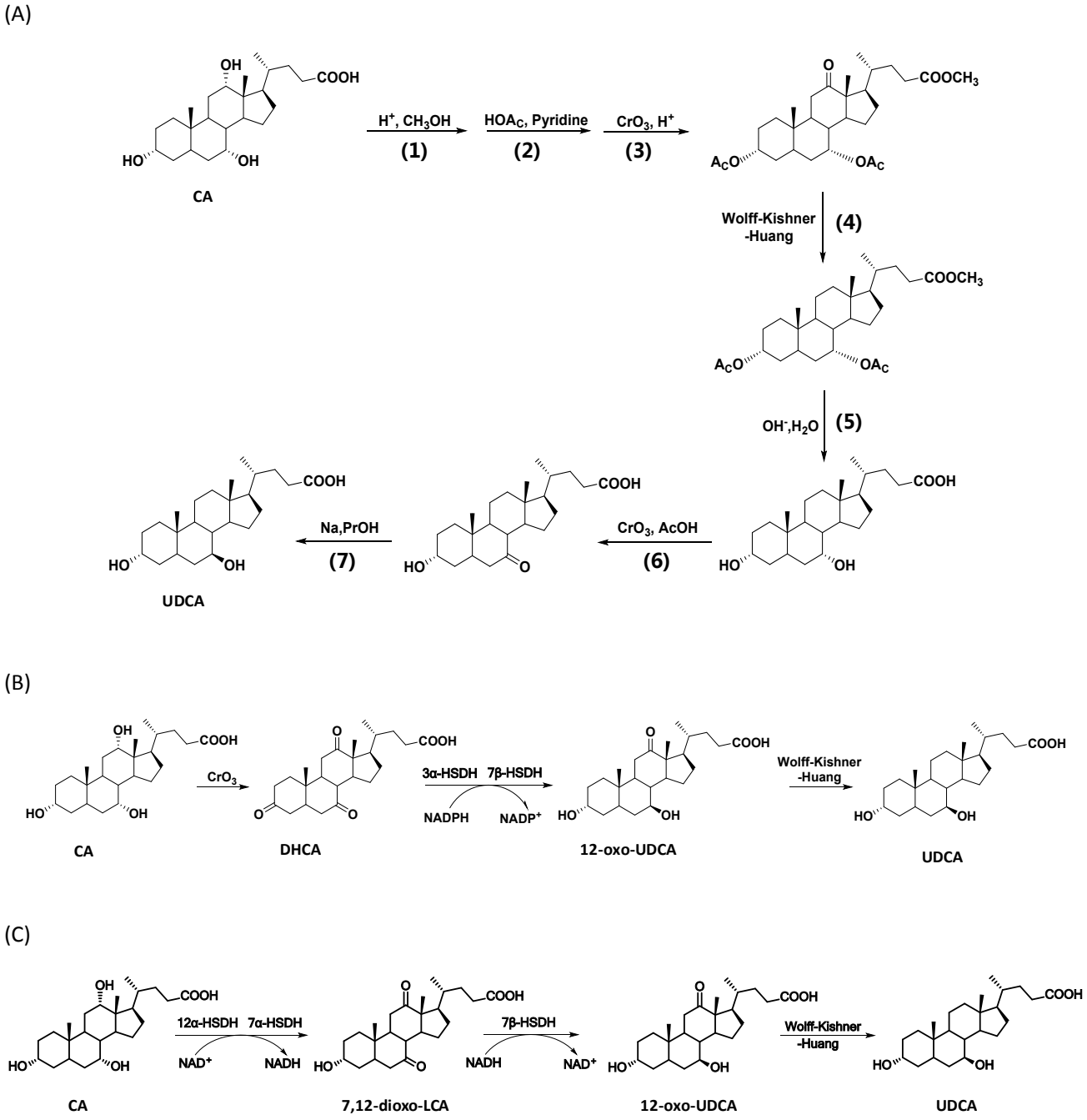


Figure S1. Synthesis of UDCA from CA by chemical (A) or chemo-enzymatic methods (B and C).

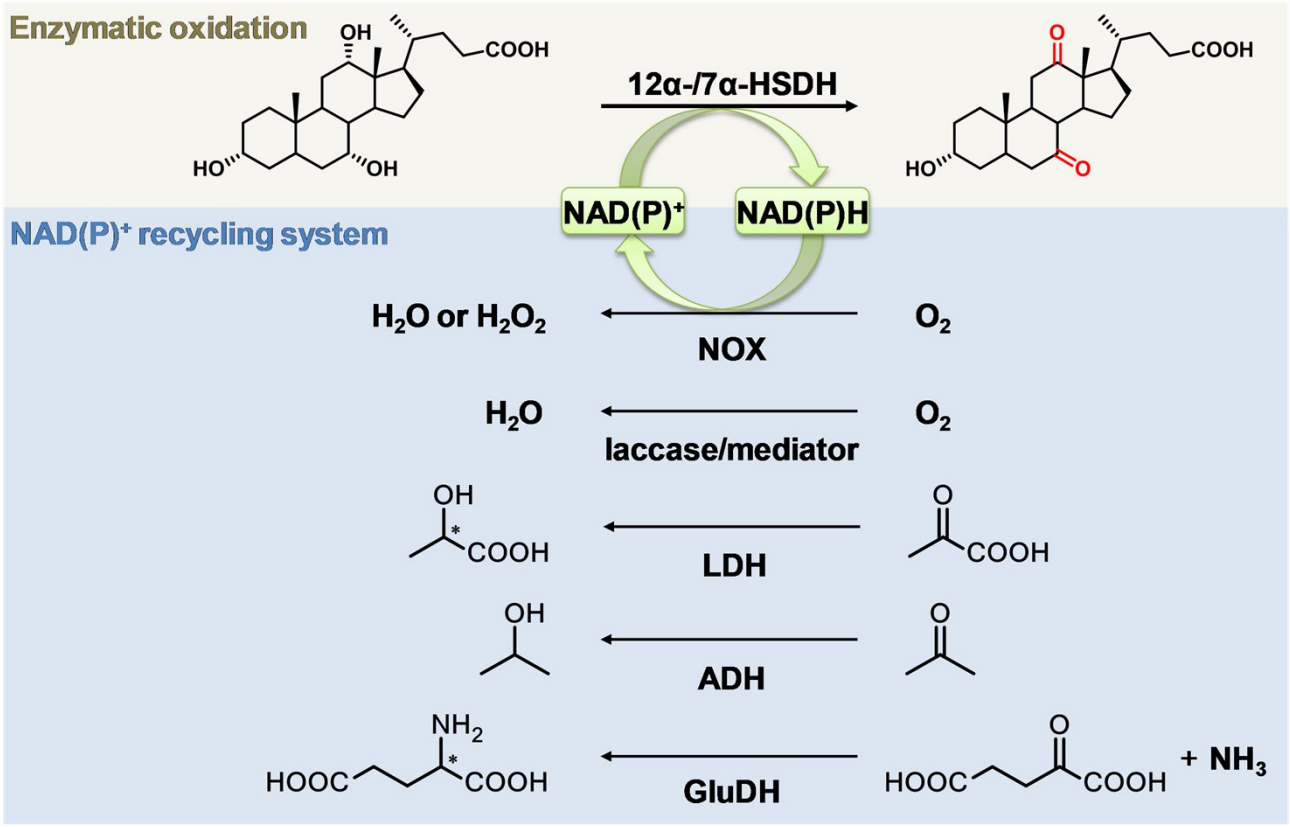


Figure S2. Currently available NAD(P)$^+$-recycling systems for the biocatalytic oxidation of CA.

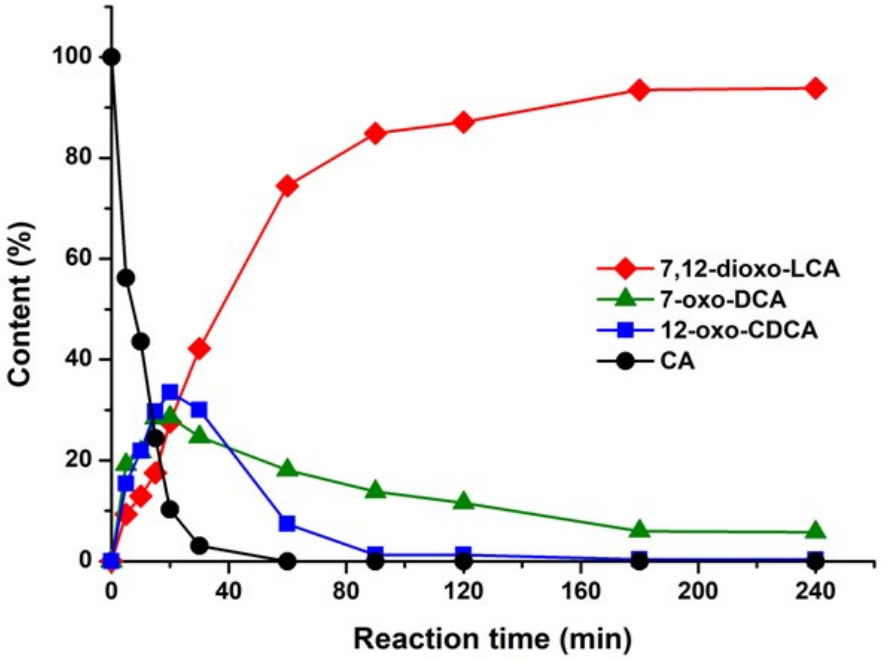


Figure S3. Reaction progress curves of the self-sufficient hydride-shuttling cascade via *in vitro* biocatalysis. Reaction conditions: 10 mL reaction mixture, containing 25 mM CA, 75 mM TMP, 0.1 mM NAD^+, lyophilized cell-free extracts of *Rr*12α-HSDH, *Ec*7α-HSDH and *Es*LeuDH (5 U mL^{-1} each), and NH_4Cl/NH_4OH buffer (1 M, pH 9.5), was incubated at 30°C and 200 rpm, the samples were withdrawn at regular intervals for HPLC analysis.

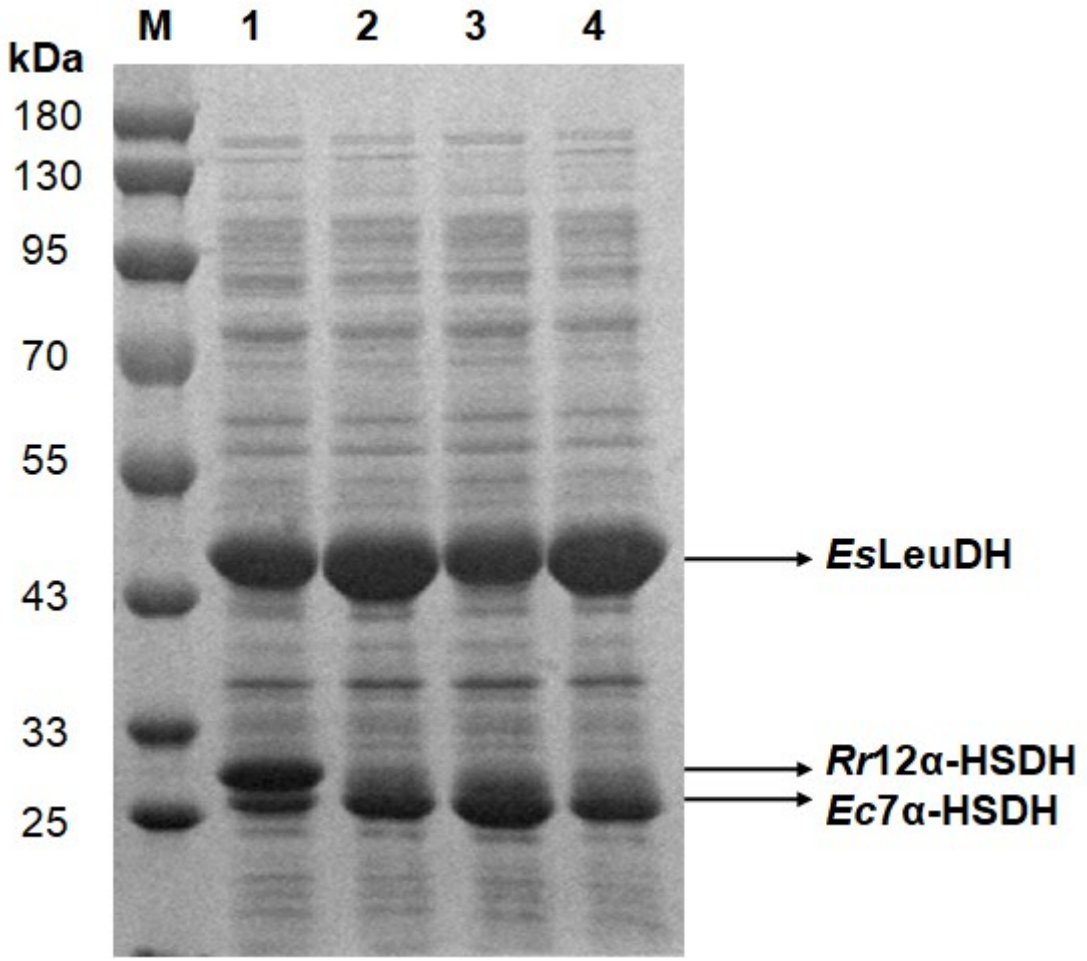


Figure S4. SDS-PAGE analysis of whole-cell proteins expressed in selected *E. coli* strains. Lane M: protein marker (kDa); Lanes 1−4 represent strain 2, strain 4, strain 6 and strain 8, respectively.

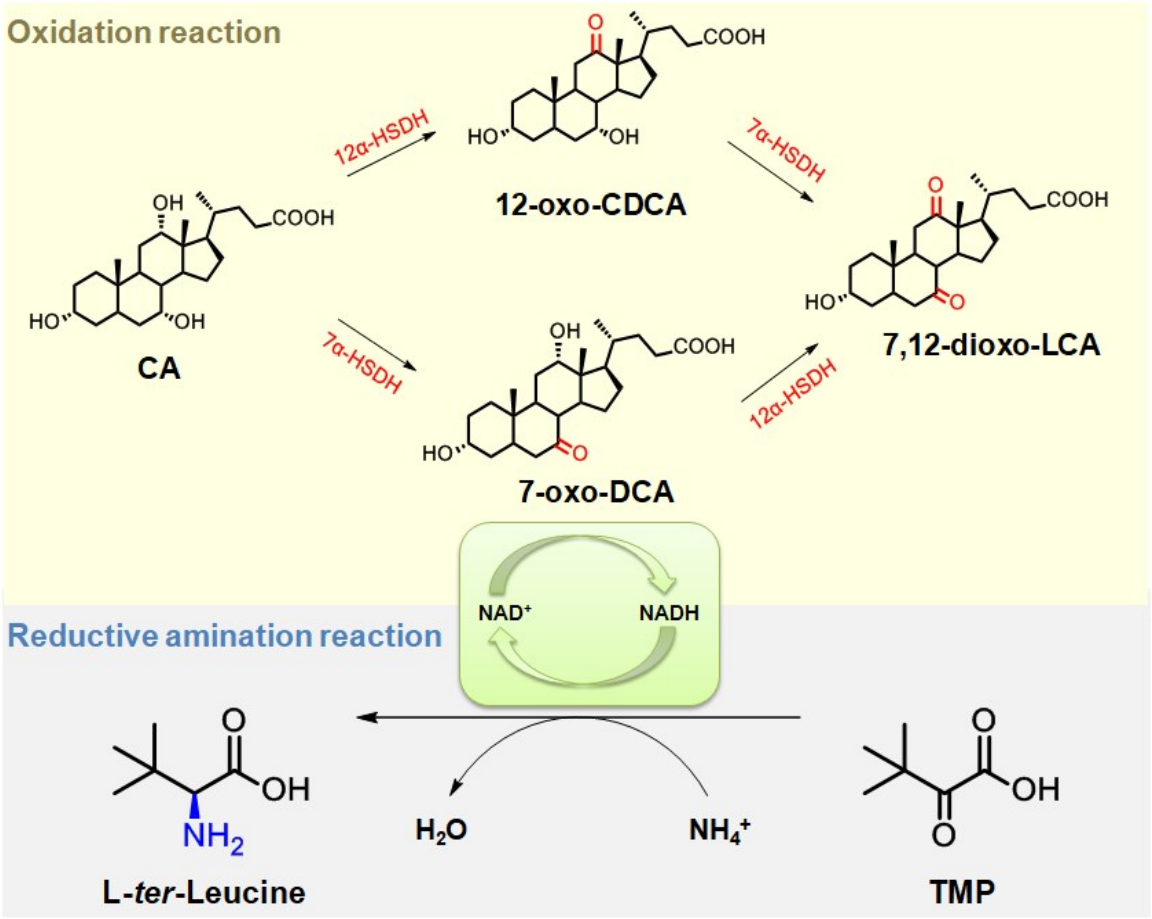


Figure S5. A detailed synthetic roadmap of the smart self-sufficient hydride-shuttling cascade for concurrently biosynthesis of 7,12-dioxo-LCA and L-*tert*-leucine.

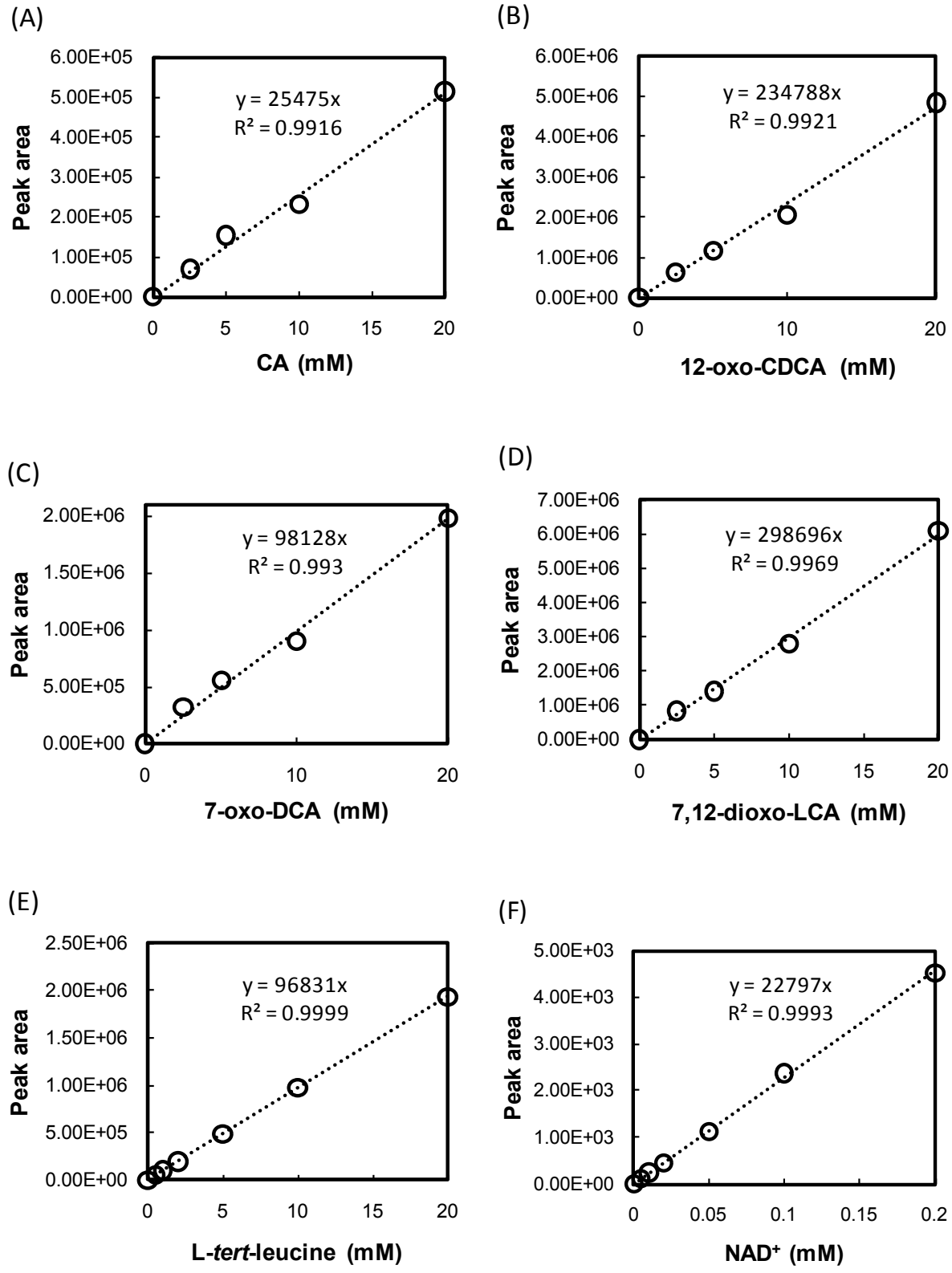
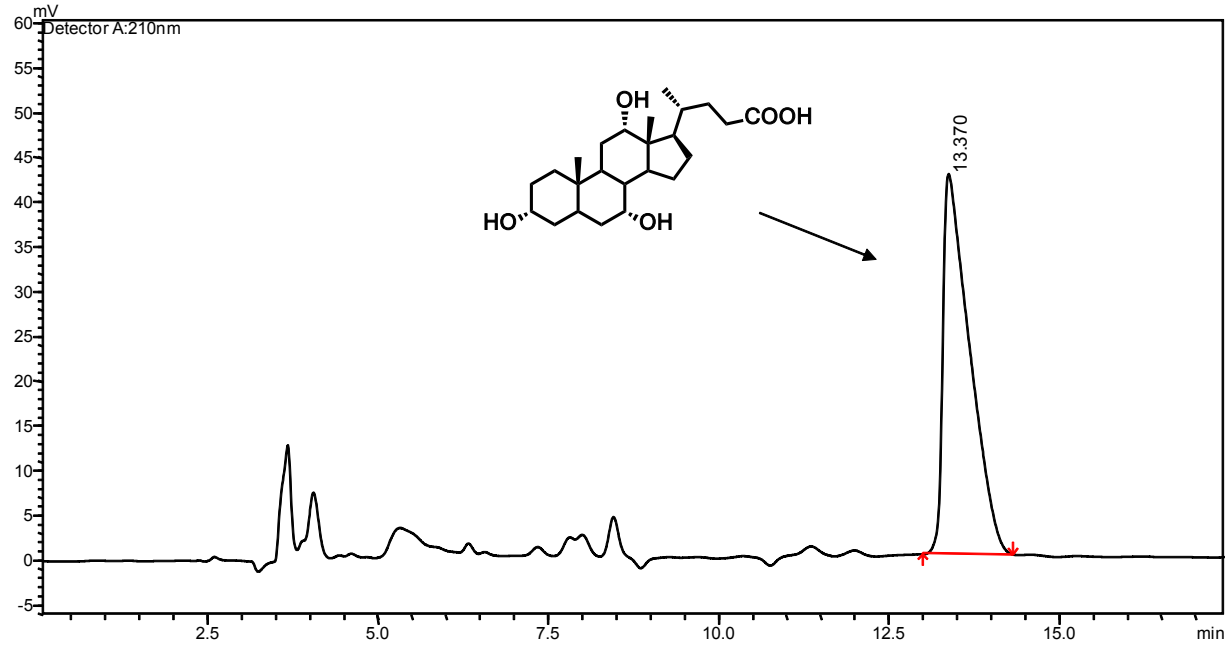
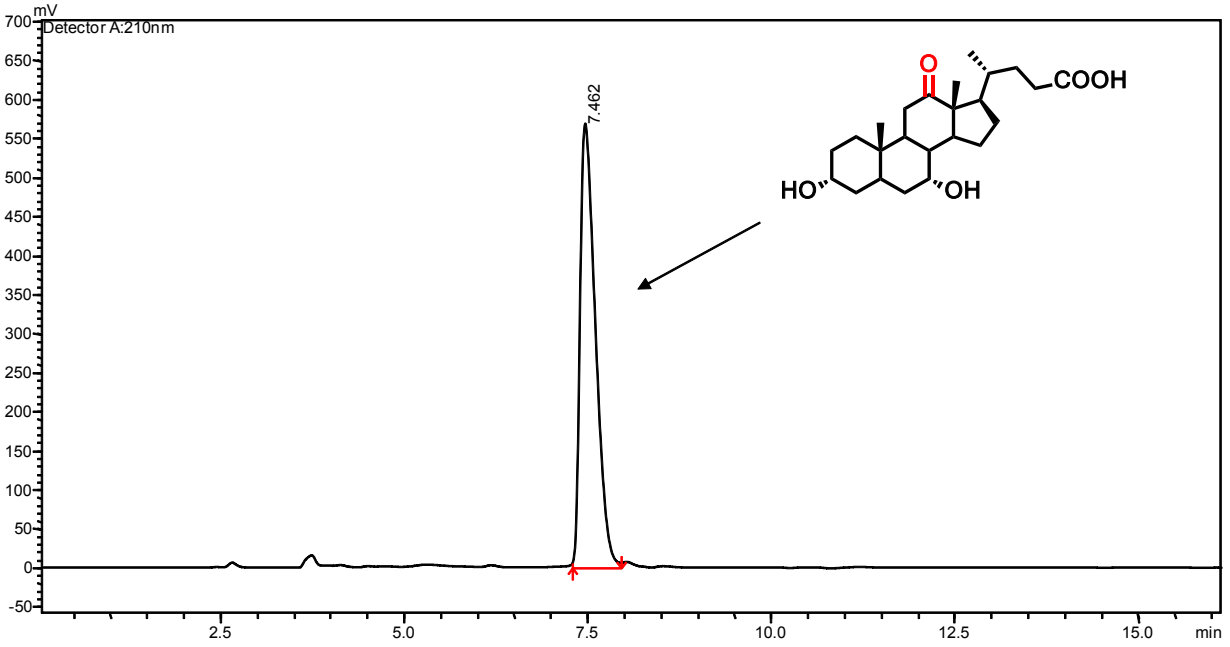


Figure S6. Calibration curves of CA (**A**), 12-oxo-CDCA (**B**), 7-oxo-DCA (**C**), 7,12-dioxo-LCA (**D**), L-tert-leucine (**E**), and NAD^+ (**F**).

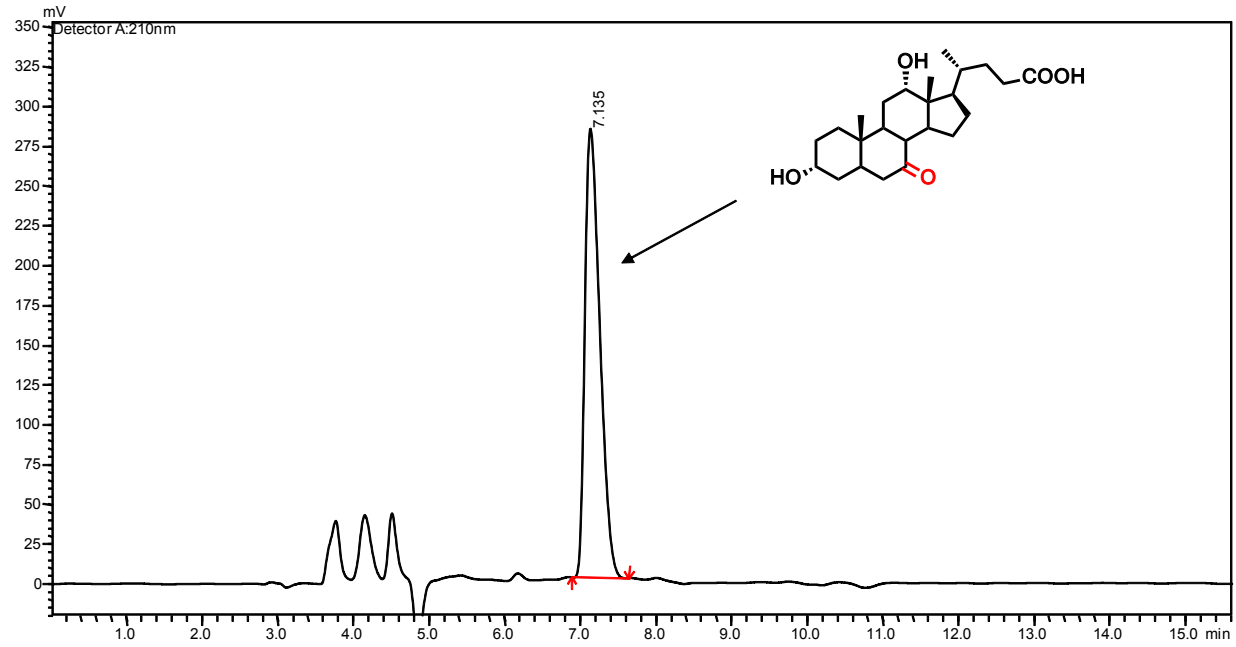
4. HPLC spectra



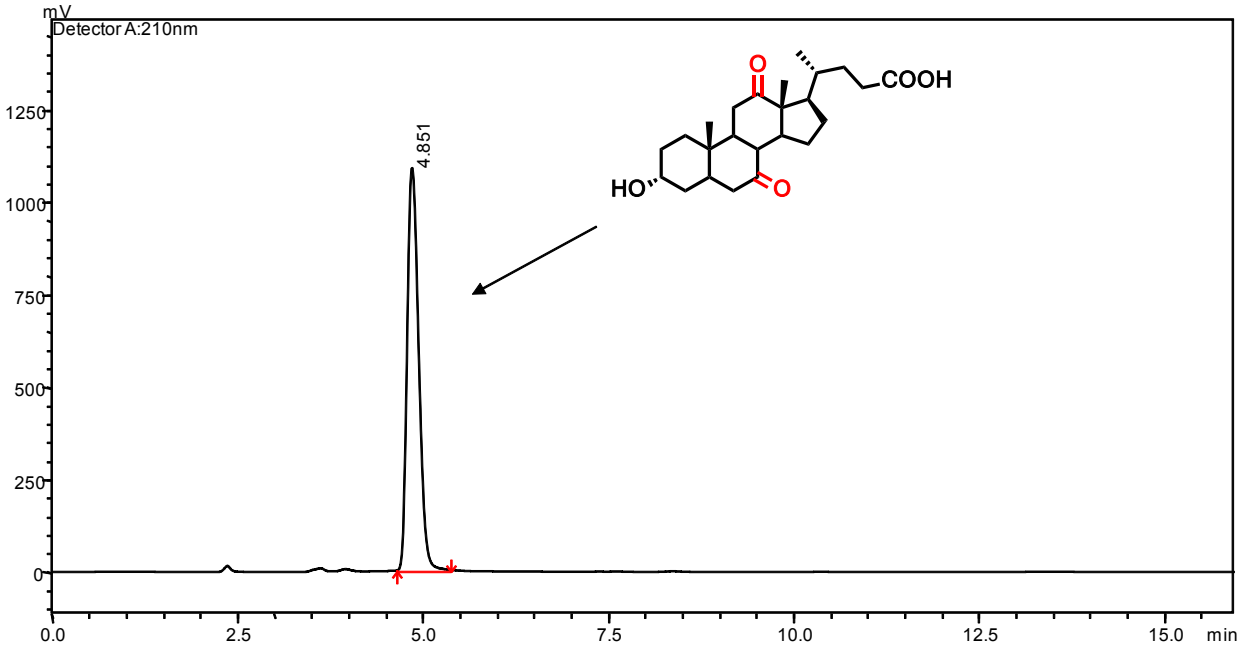
Standard CA.



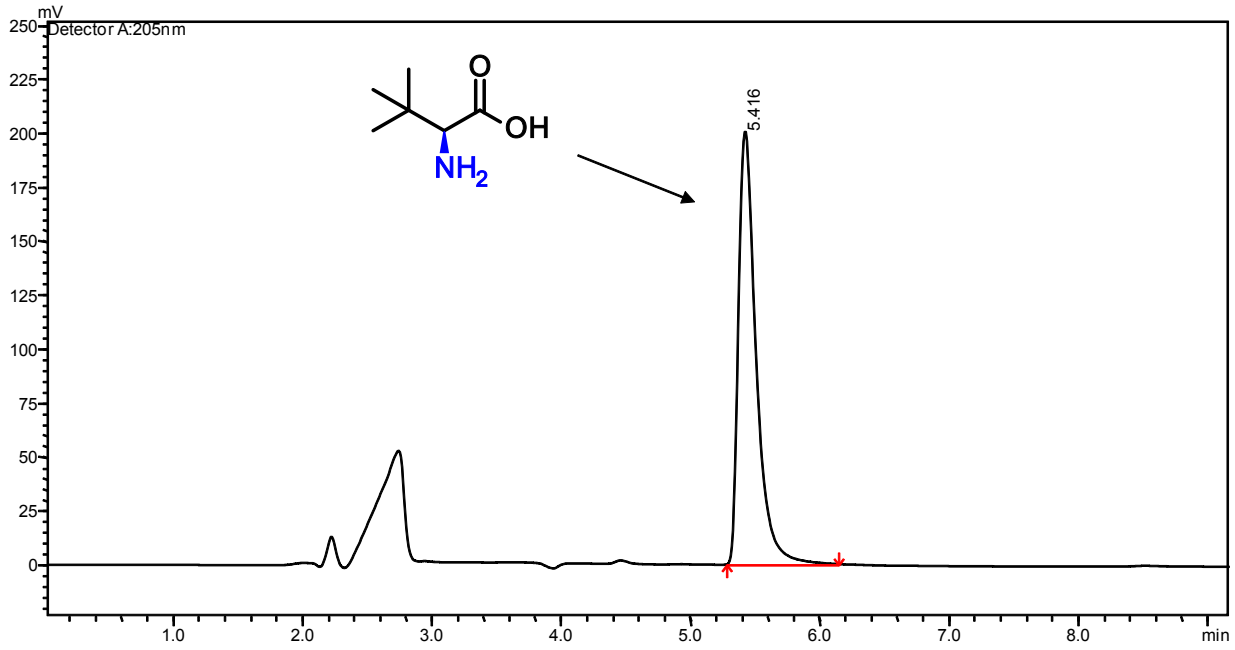
Standard 12-oxo-CDCA.



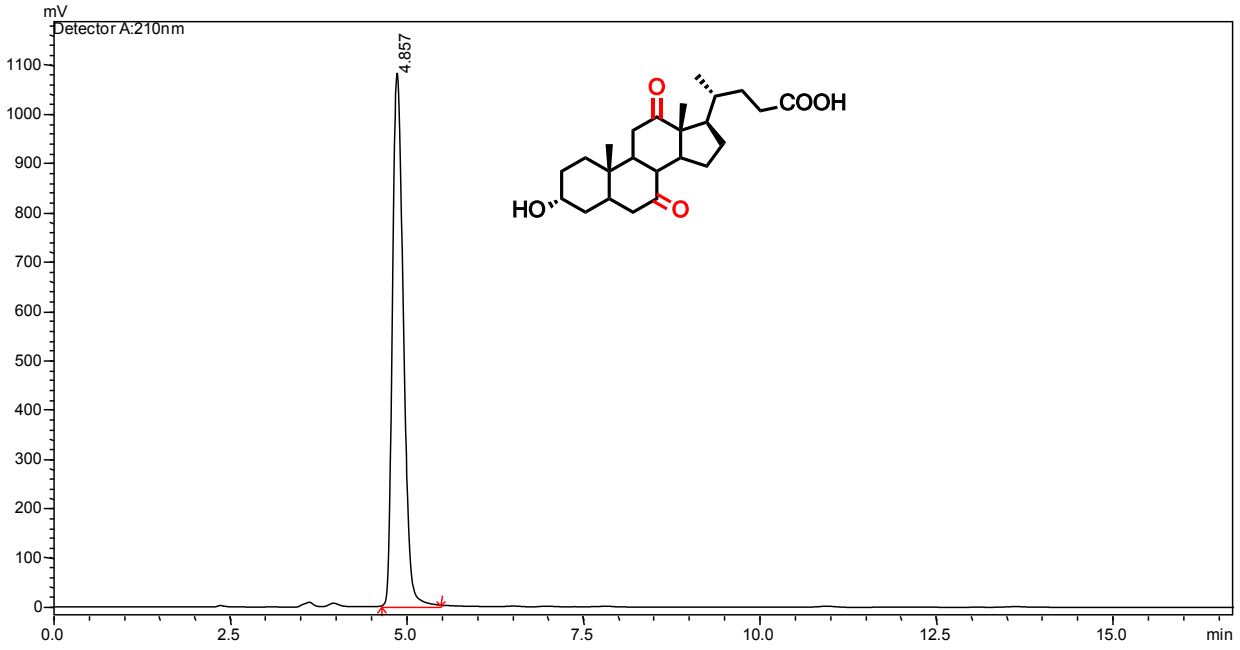
Standard 7-oxo-DCA.



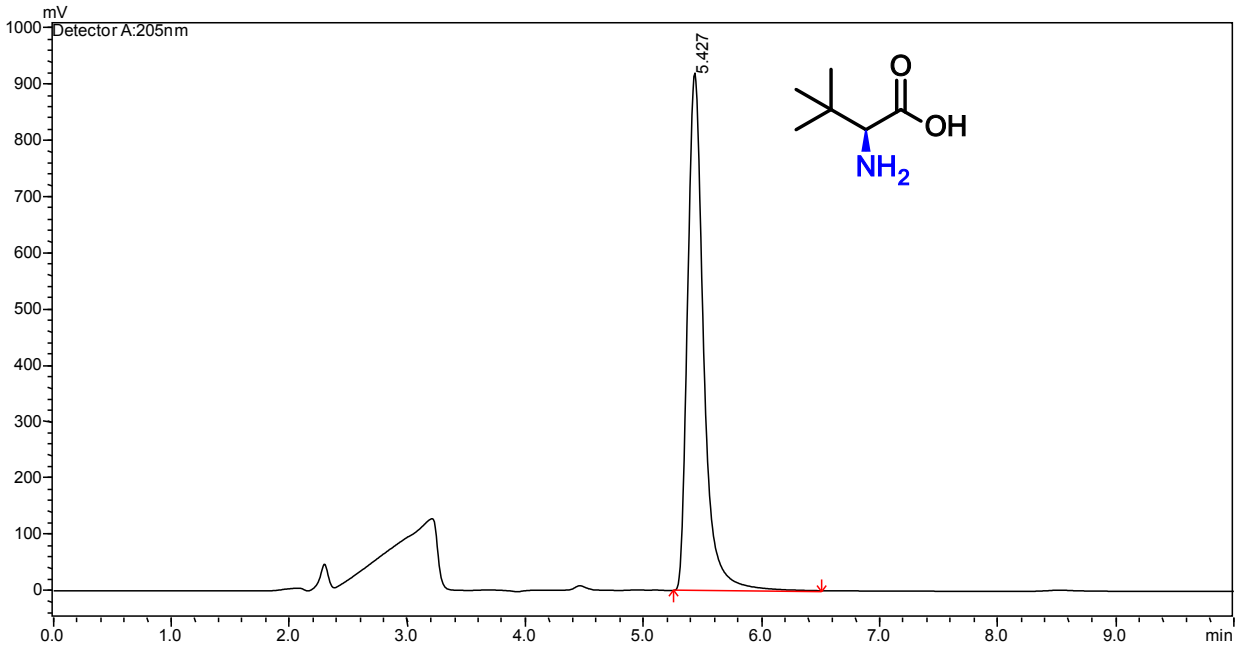
Standard 7,12-dioxo-LCA.



Standard L-*tert*-Leucine.



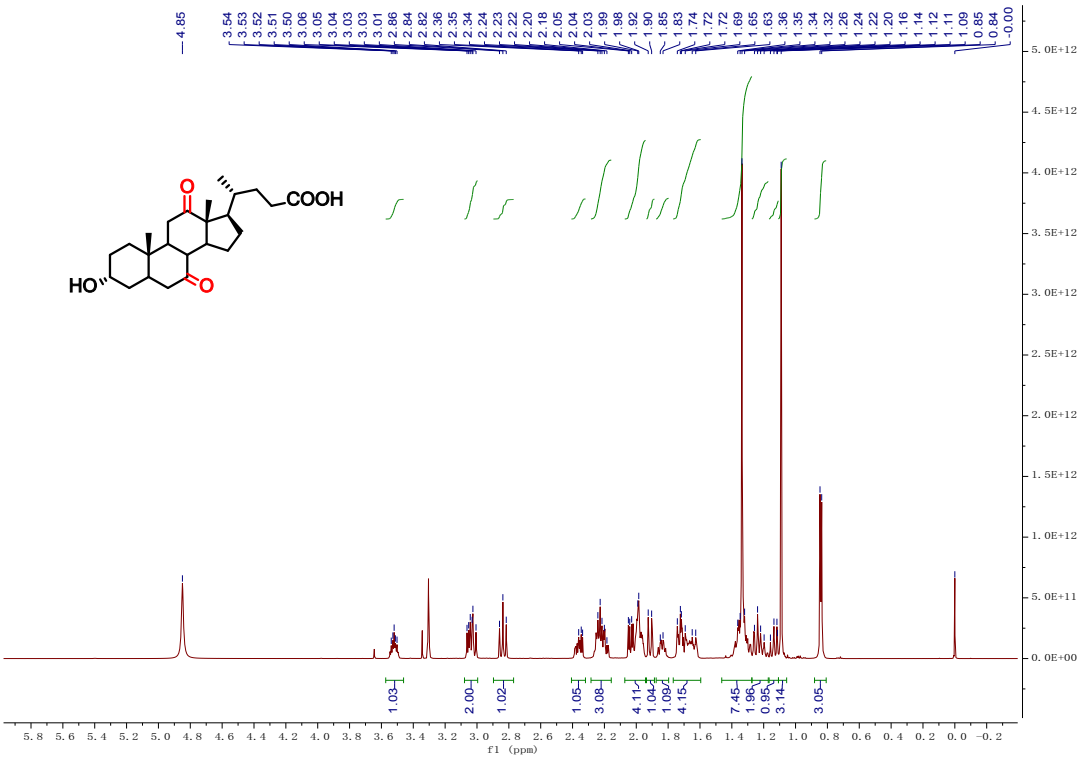
The 7,12-dioxo-LCA prepared by enzymatic cascade reaction.

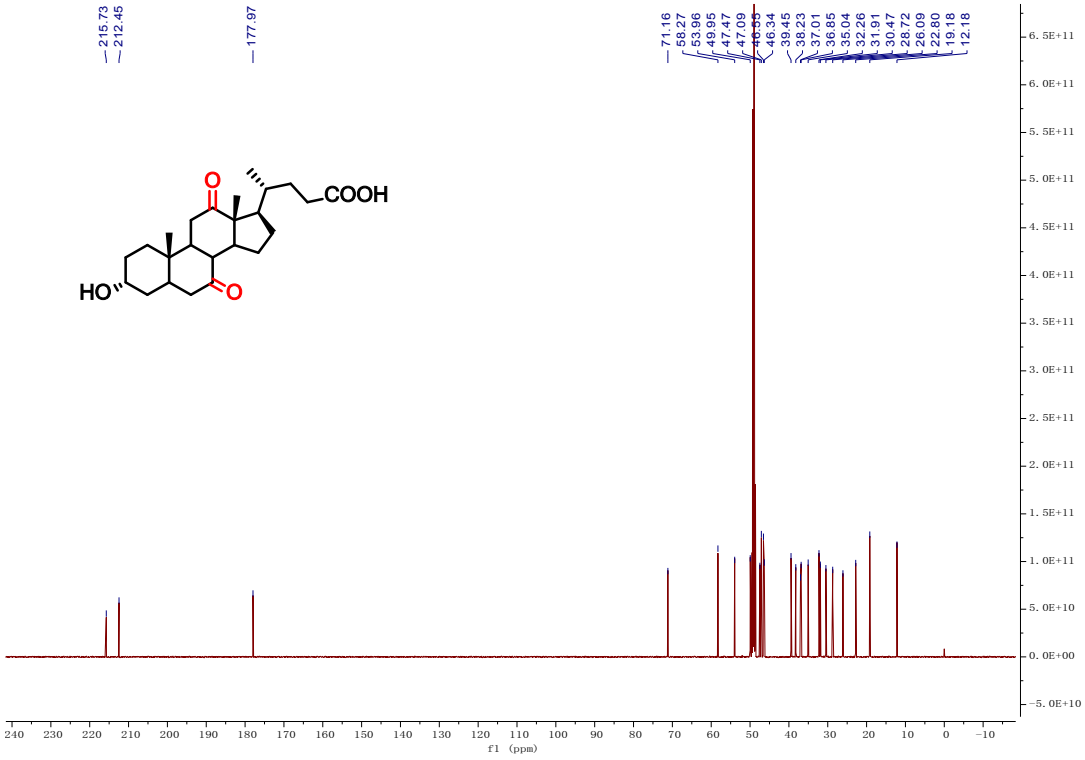


The L-*tert*-Leucine prepared by enzymatic cascade reaction.

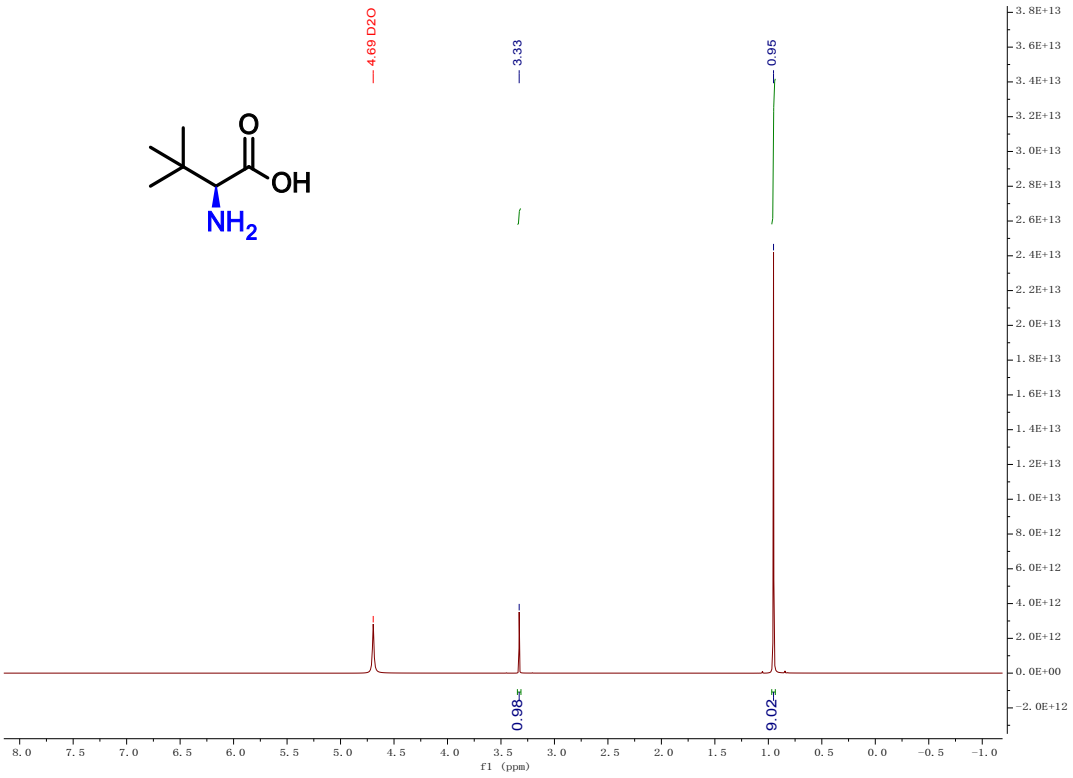
5. ^{1}H NMR and ^{13}C NMR spectra

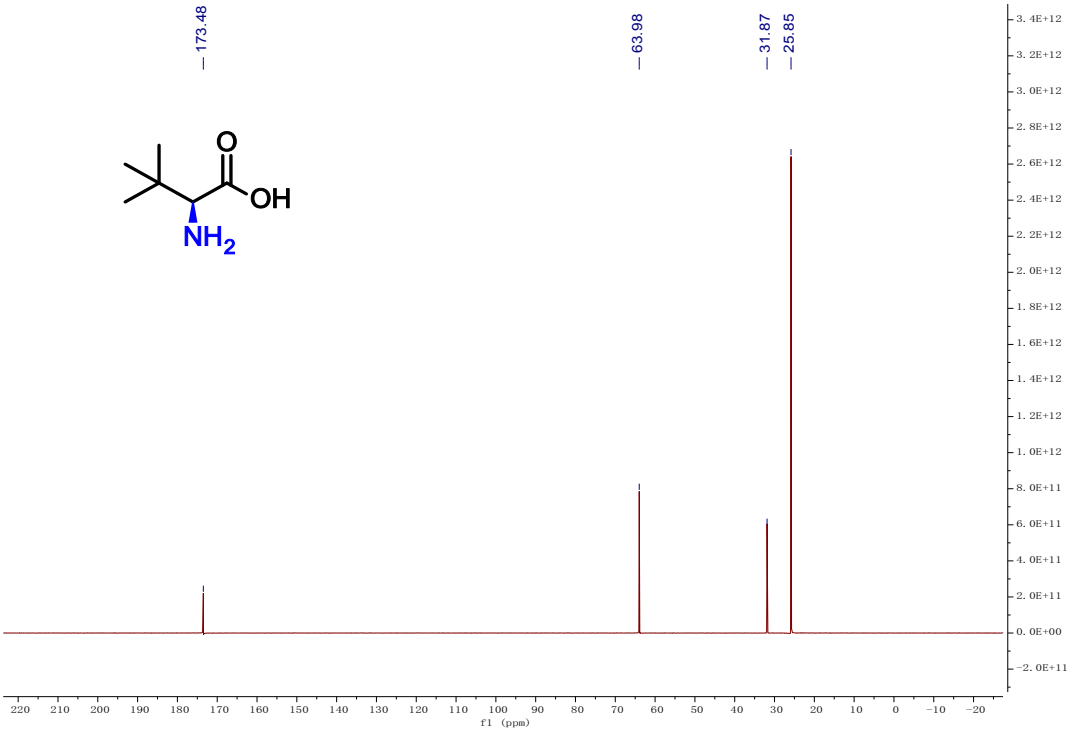
5.1 NMR of product 7,12-dioxo-LCA prepared by enzymatic cascade reaction





5.2 NMR of product L-*tert*-Leucine prepared by enzymatic cascade reaction





6. References

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