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Note

Efficient desymmetrization of 4,6-di-*O*-benzyl-*myo*-inositol by Lipozyme TL-IM

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ABSTRACT

The enantioselective enzymatic desymmetrization of 4,6-di-*O*-benzyl-*myo*-inositol, a *myo*-inositol derivative, was effectively catalyzed by *Thermomyces lanuginosus* lipase (TL-IM). The product 1D-1-*O*-acetyl-4,6-di-*O*-benzyl-*myo*-inositol, a useful precursor to inositol phosphates, was obtained in excellent yield and enantiomeric excess. Through the investigation of the effects of solvent, biocatalyst load, and temperature, a more economical procedure resulted. The feasibility of biocatalyst reuse was also shown.

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1. Introduction

Chiral *myo*-inositol derivatives, such as phosphatidylinositols and inositol phosphates (e.g., D-*myo*-inositol 1,4,5-trisphosphate) are fundamental tools for the study on cellular signaling processes.[1]

Although much development has occurred in the chemistry of inositols,[2,3] most syntheses of these relevant substances still rely on optical resolutions with chiral auxiliaries, with all disadvantages that such procedures display. The use of *myo*-inositol itself as precursor in the enantioselective syntheses of bioactive *myo*-inositols derivatives is, in many cases, rewarding. All carbon atoms in the carbocyclic backbone of that largely available, pro-chiral starting material are stereoselectively hydroxylated, which may be conducive to short synthetic routes. Nevertheless, this usually implies the need of optical resolutions of racemic intermediates.

The use of enzymes as synthetic tools for the enantioselective synthesis of natural products and other bioactive substances has gained increased attention in recent years.[4,5] In this context, lipases and other hydrolases, especially those from microbial sources, have found numerous applications,[6,7], due to high regio-, chemo- and stereoselectivies, availability, practicality, and good activity in organic solvents.[8] By means of practical kinetic resolution of racemic substances or desymmetrization of prochiral compounds, such biocatalysts allow the preparation of chiral synthetic blocks in high ee.

Few reports have dealt with the application of lipases and other hydrolases (EC 3.1.1.3) in the syntheses of inositols from *myo*-inositol itself via resolutions.[9,10] Even fewer studies were devoted to the desymmetrizations of *myo*-inositol derivatives via hydrolases.[11,12] *myo*-Inositol derivatives are interesting subjects for the study of desymmetrization protocols. Different protocols (based upon catalytic reactions or the use of chiral auxiliaries)[13,14] have been devised with this intent. Enzyme-catalyzed desymmetrizations are relevant means for the preparation of chiral substances in high chemical yields and ee.[15] Lipases and related biocatalysts are particularly effective in carrying out such transformations. Naturally, with the possibility of nearly complete conversions to the desired products, the biocatalyzed desymmetrization of inositols allows a better match between economy and practicality than the corresponding kinetic resolutions. Laumen and Ghisalba developed an efficient procedure for the desymmetrization of 4,6-di-*O*-benzyl-*myo*-inositol, **1** (Scheme 1).[11] The authors reported that Amano PS lipase (EC 3.1.1.3) (lyophilized) catalyzed this transformation in vinyl acetate, leading to the mono-*O*-acetylated compound, 1D-1-*O*-acetyl-4,6-di-O-benzyl-*myo*-inositol, D-(−)-**2**, in 89% (after recrystallization). The achieved enantioselectivity

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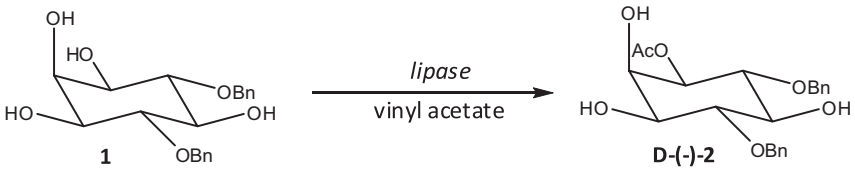


Scheme 1. Desymmetrisation of 4,6 -di-*O*-benzyl *myo*-inositol.

(ee >99%) of desymmetrization itself was not determined, as they chose to record it for the deacetylated acetal derived from D-**2**. Moreover, the reaction time of such transformations was not disclosed. Compound **1** is a precursor of relevant bioactive *myo*-inositol derivatives, such as phosphoinositides.[16]

Herein, we report on an alternative, efficient, and fast desymmetrization of inositol **1** by an immobilized, thermostable lipase. A detailed investigation on the performance of the most active lipase under different conditions and on the reaction economy was carried out. Moreover, the biocatalyst reuse was shown to be feasible, upon selection of more compatible solvents. Such features of synthesis of D-**2** have not been addressed before. Furthermore, our study established the enantioselectivity in this enzymatic transformation for the first time.

2. Results and discussion

2.1. Selection of lipases and initial analyses

Eight commercially available lipases (see experimental section) were used as biocatalysts against *myo*-inositol derivative **1** in vinyl acetate as solvent. Lipozyme TL-IM (*Thermomyces lanuginosus*) was the only enzyme to afford nearly full conversion (Fig. 1) to the acylated product. RM-IM (*Rhizomucor miehei*) and Lipomod 34P (*Candida cylindracea*) led to the monoacetylated product with low conversions (Table 1). Essentially, a single regioisomer was produced with all active enzymes. Di- and poly-acylated products were not formed.

A preparative reaction of acylation of substrate **1** under TL-IM catalysis was undertaken. The isolated product was purified and analyzed. Firstly, its ^{1}H NMR spectrum showed that the acetylation occurred at the hydroxyl group at C-1 (or C-3), thus yielding acetate **2**. Moreover, the spectrum of **2** was consistent with the data in the literature for the same compound.[11] The specific rotation of **2** indicated that the D-enantiomer, 1D-1-*O*-acetyl-4,6-di-*O*-benzyl-*myo*-inositol, (D-(−)-**2**) was produced and, by comparison to the value recorded by Ghisalba and Laumen, it suggested that a high enantioselectivity was achieved.

Table 1
Screening of lipases for the desymmetrization of **1** in vinyl acetate

Entry	Lipase	Conversion (%)[a]
1	TL-IM	95
2	RM-IM	19
3	Lipomod 34P	30

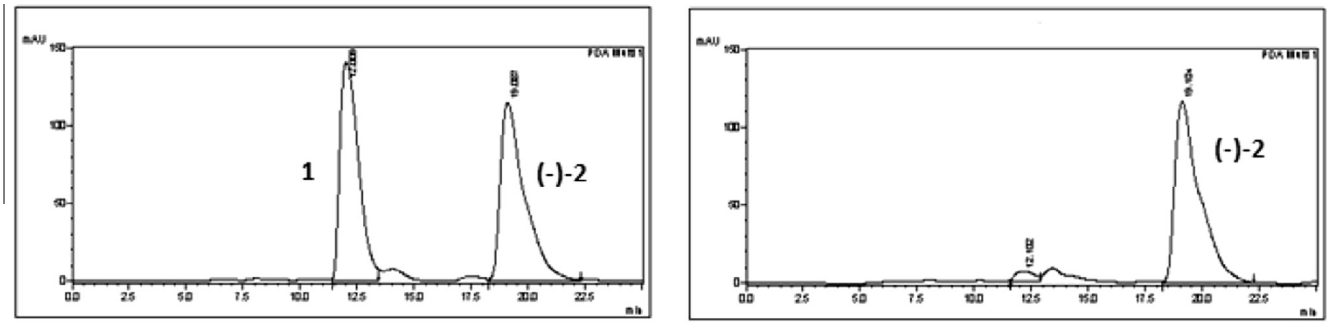
[a] 48h-reactions.

2.2. Determination of ee

The enantiomeric excesses were determined by chiral HPLC analyses (Fig. 2). All three enzymes were highly enantioselective (ee >99%; E >100). The racemic sample (DL-**2**) for these analyses was obtained by controlled acylation of tetrol **1** with Ac_2O (Et_3N, DMAP, CH_2Cl_2, −20 °C) which resulted in a mixture of mono- and polyacetates. The fraction containing the monoacetates was further resolved by HPLC, affording enough DL-**2** to develop the analytical protocol for ee determinations (Fig. 2a). These analyses showed that all lipases led to the same enantiomer, D-(−)-**2**. Due to the high performance of Lipozyme TL-IM, we focused on this lipase.

2.3. Solvent selection for the desymmetrization of *myo*-inositol derivative 1 by Lipozyme TL-IM

Organic media have a strong influence on the enzymatic activity and the enantioselectivity of lipases.[17] Therefore, we assayed the desymmetrization of **1** with vinyl acetate (1:1 ratio with solvent) by Lipozyme TL-IM in three different solvents: TBME (*tert*-butyl methyl ether), EtOAc, and hexanes (Table 2). The time course of the acylation of **1** for each condition (besides the one with vinyl acetate as solvent/acylating agent) was established. The experiment employing vinyl acetate as solvent showed that under this condition the desymmetrization of **1** was virtually complete after 10 h. High ee and E were achieved in all cases. The use of EtOAc as solvent led to lower conversions in the first hours of the reaction. However, with time, such conditions overcame the one with



(a) TL-IM-catalyzed desymmetrization of **1**, before completion.

(b) TL-IM-catalyzed desymmetrization of **1**, leading to (-)-**2** (vide Table 1)

Figure 1. HPLC analysis of conversion in the reaction of substrate **1** with vinyl acetate catalyzed by a lipase.

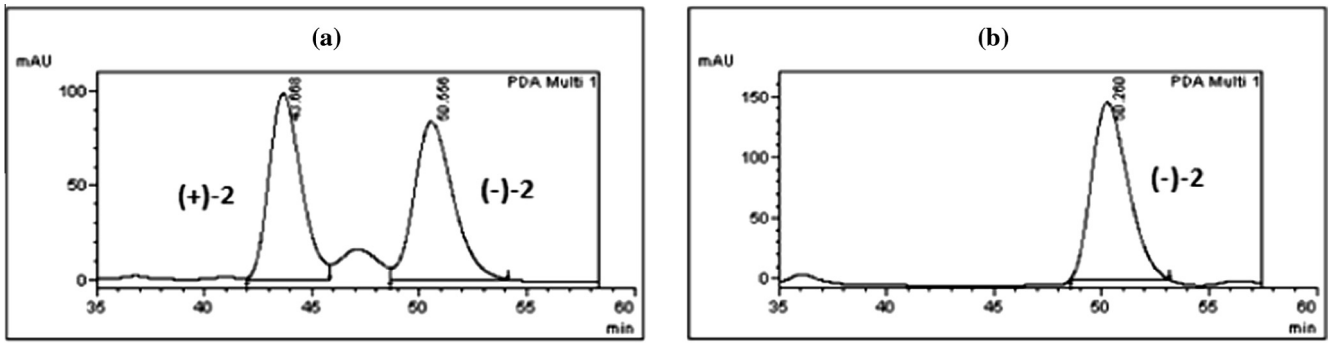


Figure 2. Chromatographic determination of *ee* for the desymmetrization of **1** in vinyl acetate by TL-IM: (a) DL-**2**, (b) D-(-)-**2**.

Table 2
Time course of conversions in the resolution of **1** catalyzed by TL-IM at 30 °C with vinyl acetate as acylating agent in different solvents

Entry	Solvents	Conversion (%)[a]					Stereoselectivity[b]	
		1 h	2 h	6 h	10 h	24 h	ee_p (%)	E
1	Vinyl acetate	55	77	95	99	95	>99	>100
2	Ethyl acetate	44	70	96	100	96	>99	>100
3	Hexane	52	88	97	100	96	>99	>100
4	TBME	86	97	97	98	96	>99	>100

[a] Formation of D-**2**.
[b] Stereoselectivity after 24 h.

Table 3
Effects of temperature and substrate/enzyme ratio on the desymmetrization of **1** by TL-IM in hexane using vinyl acetate[a]

Entry	Time (h)	45 °C[b]		60 °C[b]	
		1:3[c]	**1:5**[c]	**1:3**[c]	**1:5**[c]
1	1	55.0	95.3	83.0	97.9
2	2	88.9	95.8	94.8	99.0
3	6	95.8	95.8	99.4	100.0
4	12	99.2	100.0	98.9	99.4
5	24	95.5	100.0	98.3	98.5

[a] ee_p >99% (E >100) in all cases (after 24 h).
[b] % Conversions to D-**2** at each temperature.
[c] Inositol **1**/TL-IM ratio.

vinyl acetate as solvent. Our data show that more hydrophobic solvents (hexanes, TBME) improved the enzymatic reaction, due to substantial accelerations.

The desymmetrization of tetrol **1** in hexanes surpassed the protocol with vinyl acetate as solvent in conversion (see Section 4.5) after 6 h and was complete after 10 h. This effect was even more intense with TBME as solvent, as very high conversion (higher than that obtained with vinyl acetate as solvent after 10 h) was observed after 2 h. In all conditions, conversion erosions (related to reversal to **1**, as no by-product was detected) were observed after it reached a maximum.

The desymmetrization of **1** using ethyl acetate both as solvent and acylating agent was also attempted but no conversion was observed in 24 h. Therefore, taking into account that (compared to the reaction in vinyl acetate) less acylating agent was available in the reaction with EtOAc/vinyl acetate and that EtOAc was shown not to be effective as acylating agent, it is clear that EtOAc as solvent (in combination with vinyl acetate) had, in fact, a beneficial effect.

2.4. Effects of temperature and enzyme ratio

Lipases from *Thermomyces lanuginosus* are known for their high thermal stability.[18] Such property encouraged us to investigate the effect of temperature and enzyme concentration (substrate/enzyme ratio) on the enzymatic desymmetrization of **1** as a means to minimize the enzyme load. We chose to use hexane as solvent (instead of TBME) for economic and safety reasons. Thus, stirred mixtures of *myo*-inositol **1** and lipase TL-IM (1:3 or 1:5 mass ratios), vinyl acetate in hexane were heated to 45 °C or 60 °C and the conversions were determined at different reaction times (Table 3; Fig. 3). Increasing the temperature to 45 °C enabled a significant decrease of enzyme load. Actually, when the reaction was run at 45 °C, virtually the same conversion was obtained with a 1:3 ratio after 2 h (Table 3, entry 2) as was obtained with a 1:10 ratio in the same time (see Table 2, entry 3). Interestingly, the assays at 45 °C showed that, after 6 h, the conversion does not depend on the enzyme load (entries 3–5). At the same temperature, the reaction was essentially complete after 12 h employing only the 1:3 the ratio. On the other hand, at 60 °C, the acylation is virtually complete after 6 h and 2 h when the substrate/enzyme ratio was 1:3 and 1:5, respectively (entries 2 and 3).

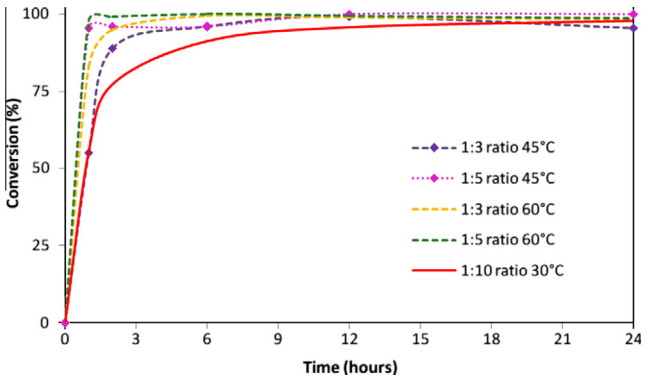


Figure 3. Time course of conversion (to D-**2**) in the desymmetrization of **1** by TL-IM at different temperatures and substrate/enzyme ratios.

Thus, higher temperatures allow lower loads of biocatalyst in the desymmetrization of inositol **1** and shorten such enantioselective transformation. These results also show the stability of TL-IM lipase is maintained in this reaction.

2.5. Reusability of Lipozyme TL-IM

The reusability of immobilized lipase is important for industrial applications since the cost of lipase can make the processes economically unattractive. Aiming at a more economical protocol, experiments were performed to examine the reusability and the stability of Lipozyme TL-IM. After every 2 h-cycle of the acylation reaction of inositol **1**, the immobilized lipase was recovered by

filtration, washed (three times) with the vinyl acetate-solvent mixture and subsequently reused. Three reuse cycles were carried out, with the conversions determined before and after each one of them (Table 4).

As shown in Table 3, the TL-IM lipase can be reused at least three times. As usual,[19] there was a gradual decrease in conversion under all conditions. Good activity was retained by TL-IM in TBME and, especially, in hexane. Taking into account the results shown in Table 3, higher conversions for the reuse experiments run in hexane at longer reaction times are expected. In fact, TL-IM, even in smaller loads and at higher temperatures, proved to be very stable over time.

Since the more hydrophilic solvents lead to steeper declines of activity, such losses may be caused by removal of water content of the catalyst. It is believed that the essential water layer on enzyme bestows flexibility, and hence, higher activity.[8] Loss of biocatalyst due to handling and mass transfer issues may also be responsible for the activity decays.[20]

3. Conclusion

We have disclosed a rapid and very efficient protocol for the desymmetrisation of *myo*-inositol derivative **1** using Lipozyme TL-IM. Thus, the desired product D-(−)-**2** was formed in excellent yield and enantiomeric excess (>99%) in a variety of solvents. The developed protocol expedites the syntheses of relevant inositol phosphates and other derivatives. It was demonstrated that the use of solvent (in combination with vinyl acetate as acylating agent) leads to a more efficient and economical transformation. The feasibility of catalyst reuse and lower enzyme load was demonstrated as well.

4. Experimental

4.1. General

Enzymes: Lipozyme TL IM (*Thermomyces lanuginosus*), Lipozyme RM IM (*Rhizomucor miehei*) and Novozyme 435 (*Candida antarctica* B, CaLB) were supplied by Novo Nordisk. AY amano 30 (*Candida rugosa*), FAP 15 amano (*Rhizopus javonicus*), G amano (*Penicillium camembertis*) and AK amano (*Peseudomonas fluorescens*) were supplied by Amano. Lipomod 34P (*Candida cylindracea*) was supplied by Biocatalysts. The substrate **1** was prepared as reported by Shashidhaŕs group.[21] HPLC grade acetonitrile, n-hexane and 2-propanol were purchased from Tedia. Vinyl acetate was purchased from Fluka.

4.2. Screening of lipases

The enzymatic reactions were realized at 30 °C in closed thermostatized flasks containing the substrate **1** (5.0 mg; 0.014 mmol), vinyl acetate (2.5 mL) as solvent and acylating agent and the lipases (50 mg). The screening reactions were monitored by thin-layer chromatography (TLC), using UV light as visualizing agent, and/or an aqueous basic solution of $KMnO_4$, and heat as developing agents. Analyses by TLC were done after 12, 24, and 48 h of the reaction.

Table 4
Reusability of TL-IM in the desymmetrization of **1** after 2 h-cycles at 30 °C using vinyl acetate and different solvents

Entry	Solvents /time (h)	2 h[b]	Reuse 1[b]	Reuse 2[b]	Reuse 3[b]
1	Vinyl acetate	79	68	52	43
2	Ethyl acetate	74	62	53	46
3	Hexane	89	82	74	69
4	TBME	95	89	74	65

[a] ee_p > 99% (E>100) in all cases (after three cycles).
[b] Conversions to D-**2** (%).

4.3. Enzyme activity assay

The Lipozyme TL-IM activity was 500.0 U/g. It was quantified by UV spectrophotometry at 410 nm from the hydrolysis reaction of *p*-nitrophenyl laurate (*p*NPL) by lipase-catalyzed product formation with the chromophore *p*-nitrophenol. A solution containing 2.5 mL of 2.5 mM *p*-nitrophenyl laurate in sodium phosphate buffer (25 mM, pH 7.0) was maintained at 30 °C for a period of 10 min. The reaction was initialized by the addition of 10 mg of the crude immobilized enzyme. One international unit (U) of *p*NPL was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPL per minute under assay conditions.

4.4. Preparation of racemic 1-*O*-acetyl-4,6-di-*O*-benzyl-*myo*-inositol

A stirred solution of 4,6-di-*O*-benzyl-*myo*-inositol (**1**) (159 mg; 0.44 mmol) and DMAP (10.8 mg; 0.088 mmol) in CH_2Cl_2 (5.0 mL) under N_2 was cooled to −20 °C and treated with Ac_2O (0.04 mL; 0.41 mmol). After 15 min, the reaction mixture was allowed to warm to 0 °C, being kept at this temperature for 10 min. Then, a saturated aqueous solution of $NaHCO_3$ (10 mL) was added to the mixture at this temperature. After 5 min, the resulting mixture was allowed to warm to room temperature. The product was extracted with AcOEt, the organic phase was dried over Na_2SO_4 and the volatiles were evaporated. Finally, the obtained residue was purified by flash chromatography (elution with ethyl acetate/hexane mixtures) to yield a white solid (53 mg), containing the monoacetate DL-**2**.

4.5. Analytical conditions

All the analyses for the determination of reaction conversion and enantiomeric excess were performed by HPLC (Pump: Shimadzu LC-20AT; Detector: Shimadzu SPD-M20A variable-wavelength UV/vis, with the detection set at 215 nm). The Shimadzu LC solution software was used for chromatogram integration.

The conversion determination (based upon the disappearance of the starting material) was performed with a C18 column eluted isocratically using acetonitrile: H_2O (40:60) mixture (flow rate 0.5 mL/min). The retention times of substrate **1** and monoacetate **L-(−)-2** were 12 min and 19 min, respectively.

Chromatographic determinations of the enantiomeric excesses was performed with a Chiralcel OD-H column (5 μm; 4.6 × 250 mm) eluted with 2-propanol:hexane (30:70) mixture (flow rate 0.8 mL/min). The retention times of monoacetates D-(+)-**2** and L-(−)-**2** were 43.6 min and 50.5 min, respectively.

4.6. Solvent selection for the desymmetrization of *myo*-inositol derivative 1 by Lipozyme TL-IM

The assays were performed according to the same protocol of Section 4.2 (screening of lipases), run at 30 °C, employing **1** (5.0 mg), TL-IM (50.0 mg), vinyl acetate (1.25 mL), and solvent (1.25 mL) at 30 °C.

HPLC analyses of conversion were done at reactions times of 1, 2, 6, 10, and 24 h, by independent experiments. HPLC determinations of enantiomeric excess were done after 24 h.

4.7. Reusability of Lipozyme TL-IM

The reusability of Lipozyme TL-IM was studied under the same conditions as described in Section 4.6 (solvent selection). After

each 2 h-reaction cycle, the lipase was recovered by filtration and washed three times with vinyl acetate (1.0 mL) and solvent (1.0 mL) solution to remove any remaining substrate and product before the next experiment. The filtrate produced in the last washing was analyzed to secure that the substrate **1** and D-**2** did not remain in the recovered catalyst sample. This procedure was repeated three times in the four different media to examine the extent of the stability of the immobilized enzyme.

4.8. Effects of temperature and enzyme ratio

The assays were performed according to the same protocol of Section 4.2 (screening of lipases), run at 30 °C, employing **1** (5.0 mg), TL-IM (15 or 25 mg), vinyl acetate (1.25 mL), and hexane (1.25 mL) at 45 or 60 °C.

HPLC analyses of conversion were done in 1, 2, 6, 10, and 24 h and HPLC analyses of enantiomeric excess were done in 24 h.

4.9. Preparative desymmetrisation of 4,6-di-*O*-benzyl-*myo*-inositol (1)

Lipase TL-IM (92mg, Lot. N° 10652b) was added to a solution of **1** (30.7 mg; 0.085 mmol) in a mixture of vinyl acetate: hexane (7.7 mL: 7.7 mL). The suspension stirred at 45 °C for 12 h. The enzyme was filtered of through a silica plug and the filtrate volatiles were evaporated to yield D-(−)-**2** (33.9 mg; 99% yield). HPLC and NMR analyses of the crude product showed no contamination by regioisomers. D-(−)-**2**: mp: 96–97 °C; $[\alpha]_D^{20}$ −39.3 (*c* 1.00, MeOH) Lit.[11] $[\alpha]_D^{20}$ −39.1 (*c* 1.00, MeOH), R_f = 0.45 (CH_2Cl_2/MeOH = 9:1); IR *v* (KBr) cm^{-1}: 3460, 3100, 3060, 3030, 2919, 2881, 1740, 1490, 1450, 1360, 1249, 1238, 1130, 1111, 1090, 1070, 1050,900, 730, 700.; ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.26 (m, 10H); 4.92–4.83 (dd, 4H); 4.14(s, 1H), 3.69–3.65 (t, 2H); 3.57–3.53(m, 3H); 2.67 (s, 1H); 2.52–2.50 (t, 2H). ^{13}C NMR (200 MHz, $CDCl_3$) δ 169.8; 138.9; 137.9; 137.7; 128.1; 127.7; 124.4; 127.2; 109.8; 80.8; 79.0; 78.4; 73.8; 73.5; 73.3; 72.9; 70.6; 27.8; 25.2; 20.7.

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