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Lipase-catalyzed regioselective synthesis of lipophilic inosine ester derivatives

Na Wang, Qi Wu, Wei Bo Wu, Jing Quan, Xian Fu Lin *

Department of Chemistry, Zhejiang University, Hangzhou 310027, PR China

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Abstract

Enzymatic synthesis of fatty acid inosine esters was performed by the immobilized lipase from *Mucor miehei* (Lipozyme®)-catalyzed transesterification reaction of inosine and vinyl fatty acid esters (from vinyl caprylate to vinyl stearate) in acetone. Inosine was regioselectivly acylated at the primary hydroxyl groups and inosine derivatives with long chain acyl group were prepared in good yields. Reaction conditions including enzyme resources and solvents for the esterification were examined. The obtained 5′-*O*-acyl derivatives are more lipophilic than the parent inosine and thus suitable for potentially pharmaceutical application.

Keywords: Enzymatic synthesis; Regioselectivity; Immobilized lipase; Inosine ester

1. Introduction

Nucleosides and nucleoside analogues are fundamental building blocks of biological systems and have shown remarkable antiviral and antitumor activities [1,2]. Consequently, extensive modifications have been made to both the heterocyclic base and sugar moiety in order to improve their activities for certain applications.

However, most of the conventional chemical modifications of nucleosides containing multiple hydroxyl groups need process of protection/deprotection and rigorous reaction conditions [3]. Thus, application of biocatalysts has become an important method to synthesize nucleoside derivatives due to simple feasibility and high selectivity [4,5]. Riva et al. were the first to demonstrate regioselectively enzymatic acylation of nucleosides, using trichloroethyl butyrate in DMF [6]. Gotor and co-workers reported that oxime esters were useful acyl donors for acylation of nucleosides such as thymidine, uridine and inosine by lipases [7–9]. They also reviewed the utility of biocatalysts for the modification of nucleosides, carbocyclic nucleosides, and C-nucleosides [10]. Hanson et al. reported the lipase-catalyzed synthesis of lobucavir prodrug, which is a nucleoside analogue as an antiviral agent for treatment of herpes viruses and hepatitis B [11].

Inosine [9-β-D-ribofuranosylhypoxanthine] is one of the basic compounds comprising cells, and plays a supportive role in the body [12]. Herein, we wish to report an effective and convenient enzymatic way to synthesize inosine fatty acid ester. Immobilized lipase from *Mucor miehei* was selected as enzyme catalyst and acetone was chosen as solvent (Scheme 1). A series of inosine esters with long chain alkyl group were regioselectivly prepared in moderate yields. The obtained inosine fatty acid esters are more lipophilic than the parent inosine and thus suitable for potentially pharmaceutical application.

2. Experimental

2.1. Materials

Immobilized lipase from *M. miehei* (Lipozyme®), lipase from *Mucor javanicus* and lipase from *Candida cylindracea* were purchased from Fluka. Lipase from porcine pancreas and lipase from *Candida rugose* were purchased from Sigma.

* Corresponding author. Tel.: +86 571 87953001; fax: +86 571 87952618.
E-mail address: llc123@zju.edu.cn (X.F. Lin).

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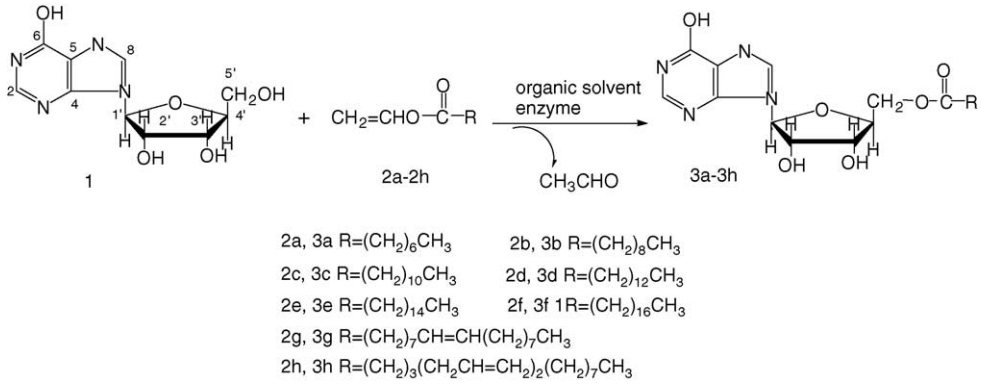

Scheme 1. Enzyme catalyzed synthesis of inosine esters in organic solvents.

Alkaline protease from *Bacillus subtilis* was purchased from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). Solvents were dried over 3 Å molecular sieves for 24 h prior to use. Analytical grade inosine was kindly provided by Zhaoqing Guangdong Star Lake Bioscience Co. Inc. (Zhaoqing, P.R. China). All other chemicals were of the highest purity commercially available.

2.2. Analytical methods

The reactions were monitored by TLC on silica gel plates with ethyl acetate/methanol/water (17:3:1, v/v). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-500 MHz spectrometer, using DMSO-d_6 as a solvent and TMS as internal reference. The yields of reaction were analyzed by Agilent 1100 HPLC with a DAD detector and a reversed-phase Shim-Pack VP-ODS column (150 mm × 4.6 mm), mobile phase was performed with a mixture of acetonitrile/water (65:35, v/v) at 1 ml min^{-1}.

2.3. Synthesis of vinyl fatty acid esters

Vinyl fatty acid esters were synthesized and purified as described by Yang et al. [13]. Carboxylic acid (0.05 mol) and mercuric acetate (0.006 mol) were dissolved in 150 ml vinyl acetate. After stirring the mixture for 30 min at room temperature, 0.5 ml of concentrated H_2SO_4 was drop-wised and the solution was refluxed for 6 h. The mixture was then cooled to room temperature and sodium acetate 5 g was added to quench the catalyst. The resulting mixture was filtered and the filtrate was concentrated under reduced pressure. The crude products were purified by silica gel column chromatography (petroleum ether/ethyl acetate 20:1, v/v).

2.4. Synthesis of 5′-O-capryloylinosine (**3a**)

The reaction was initiated by adding 10 mg/ml Lipozyme® to 25 ml acetone containing inosine (2.5 mmol), vinyl caprylate (10 mmol). The suspension was kept at 50 °C and stirred at 250 rpm for 24 h. The reaction was terminated by filtering of the enzyme and acetone was evaporated. Formation of inosine ester was monitored by TLC. The product was purified by silica gel chromatography with an eluent consisting of ethyl acetate/methanol/water (20:1:0.8, v/v). The product yield was 62%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.41 (br, 1H, 6-OH), 8.26 (s, 1H, 2-H), 8.07 (s, 1H, 8H), 5.89 (d, 1H, J = 4.8 Hz, 1′-H), 5.62 (s, 1H, 3′-OH), 5.38 (s, 1H, 2′-OH), 4.55 (t, 1H, J = 5.1 Hz, 3′-H), 4.32 (m, 1H, 2′-H), 4.19 (m, 2H, 4′-H, 5′-H), 4.08 (m, 1H, 5′-H), 2.29 (t, 2H, J = 7.3 Hz, –CH_2CO), 1.49 (m, 2H, –CH_2–), 1.22 (m, 8H, 4-CH_2–), 0.83 (t, 3H, J = 6.9 Hz, –CH_3).

2.5. Synthesis of 5′-O-caprinoylinosine (**3b**)

5′-*O*-Caprinoylinosine was synthesized by the same method as for 5′-*O*-capryloylinosine. Here, the reaction time was 48 h and the yield of the product was 60%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.38 (br, 1H, 6-OH), 8.25 (s, 1H, 2-H), 8.06 (s, 1H, 8-H), 5.88 (d, 1H, J = 4.9 Hz, 1′-H), 5.59 (d, 1H, J = 5.8 Hz, 3′-OH), 5.36 (d, 1H, J = 5.5 Hz, 2′-OH), 4.54 (t, 1H, J = 5.3 Hz, 3′-H), 4.32 (m, 1H, 2′-H), 4.19 (m, 2H, 4′-H, 5′-H), 4.08 (t, 1H, J = 4.6 Hz, 5′-H), 2.30 (t, 2H, J = 5.9 Hz, J = 7.3 Hz, –CH_2CO), 1.49 (m, 2H, –CH_2–), 1.25 (m, 12H, 6-CH_2–), 0.84 (t, 3H, J = 7.1 Hz, –CH_3).

2.6. Synthesis of 5′-O-lauroylinosine (**3c**)

5′-*O*-Lauroylinosine was synthesized by the same method as for 5′-*O*-caprinoylinosine. The yield of product was 65%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.40 (br, 1H, 6-OH), 8.26 (s, 1H, 2-H), 8.06 (s, 1H, 8-H), 5.88 (d, 1H, J = 4.9 Hz, 1′-H), 5.61 (d, 1H, J = 5.7 Hz, 3′-OH), 5.38 (d, 1H, J = 5.4 Hz, 2′-OH), 4.54 (d, 1H, J = 5.2 Hz, 3′-H), 4.31 (m, 1H, 2′-H), 4.19 (m, 2H, 4′-H, 5′-H), 4.09 (t, 1H, J = 4.5 Hz, J = 4.4 Hz, 5′-H), 2.30 (m, 2H, –CH_2CO), 1.48 (m, 2H, –CH_2–), 1.25 (m, 16H, 8-CH_2–), 0.84 (t, 3H, J = 6.7 Hz, –CH_3).

2.7. Synthesis of 5′-O-myristoylinosine (**3d**)

5′-*O*-Myristoylinosine was synthesized by the same method as for 5′-*O*-caprinoylinosine. The yield of product was 62%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.38 (br, 1H,

6-OH), 8.26 (s, 1H, 2-H), 8.07 (s, 1H, 8-H), 5.88 (d, 1H, J = 4.9 Hz, 1′-H), 5.61 (d, 1H, J = 5.7 Hz, 3′-OH), 5.38 (d, 1H, J = 5.4 Hz, 2′-OH), 4.55 (d, 1H, J = 5.2 Hz, 3′-H), 4.30 (m, 1H, 2′-H), 4.20 (m, 2H, 4′-H, 5′-H), 4.08 (m, 1H, 5′-H), 2.30 (m, 2H, $-CH_2CO$), 1.48 (m, 2H, $-CH_2-$), 1.25 (m, 20 H, 10-CH_2-), 0.85 (t, 3H, J = 6.8 Hz, $-CH_3$).

2.8. Synthesis of 5′-O-palmitoylinosine (**3e**)

5′-*O*-Palmitoylinosine was synthesized by the same synthesis method as for 5′-*O*-capryloylinosine. The reaction time was 72 h. The yield of product was 63%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.41 (br, 1H, 6-OH), 8.26 (s, 1H, 2-H), 8.07 (s, 1H, 8-H), 5.89 (d, 1H, J = 4.9 Hz, 1′-H), 5.61 (d, 1H, J = 5.7 Hz, 3′-OH), 5.38 (d, 1H, J = 5.4 Hz, 2′-OH), 4.55 (d, 1H, J = 5.2 Hz, 3′-H), 4.31 (m, 1H, 2′-H), 4.20 (m, 2H, 4′-H, 5′-H), 4.09 (t, 1H, J = 4.3 Hz, 5′-H), 2.30 (t, 2H, J = 6.9 Hz, $-CH_2CO$), 1.48 (m, 2H, $-CH_2-$), 1.24 (m, 24H, 12-CH_2-), 0.85 (t, 3H, J = 6.7 Hz, $-CH_3$).

2.9. Synthesis of 5′-O-stearoylinosine (**3f**)

5′-*O*-Stearoylinosine was synthesized by the same synthesis method as for 5′-*O*-palmitoylinosine. The yield of product was 66%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.38 (br, 1H, 6-OH), 8.25 (s, 1H, 2-H), 8.06 (s, 1H, 8-H), 5.89 (d, 1H, J = 4.9 Hz, 1′-H), 5.59 (d, 1H, J = 5.7 Hz, 3′-OH), 5.36 (d, 1H, J = 5.4 Hz, 2′-OH), 4.55 (d, 1H, J = 5.2 Hz, 3′-H), 4.30 (m, 1H, 2′-H), 4.19 (m, 2H, 4′-H, 5′-H), 4.09 (t, 1H, J = 4.5 Hz, J = 4.3 Hz, 5′-H), 2.30 (m, 2H, $-CH_2CO$), 1.48 (m, 2H, $-CH_2-$), 1.23 (m, 28H, 14-CH_2-), 0.85 (t, 3H, J = 6.5 Hz, $-CH_3$).

2.10. Synthesis of 5′-O-oleoylinosine (**3g**)

5′-*O*-Oleoylinosine was synthesized by the same synthesis method as for 5′-*O*-palmitoylinosine. The yield of product was 49%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.35 (br, 1H, 6-OH), 8.25 (s, 1H, 2-H), 8.06 (s, 1H, 8-H), 5.89 (d, 1H, J = 4.8 Hz, 1′-H), 5.59 (d, 1H, J = 5.7 Hz, 3′-OH), 5.31 (m, 3H, $-CH=CH-$, 2′-OH), 4.54 (t, 1H, J = 4.9 Hz, 3′-H), 4.31 (m, 1H, 2′-H), 4.19 (m, 2H, 4′-H, 5′-H), 4.08 (t, 1H, J = 4.5 Hz, 5′-H), 2.29 (t, 2H, J = 7.1 Hz, $-CH_2CO$), 1.98 (m, 4H, 2–CH_2-), 1.48 (m, 2H, $-CH_2-$), 1.22 (m, 20H, 10-CH_2-), 0.84 (t, 3H, J = 6.5 Hz, $-CH_3$).

2.11. Synthesis of 5′-O-linoleoylinosine (**3h**)

5′-*O*-Linoleoylinosine was synthesized by the same synthesis method as for 5′-*O*-palmitoylinosine. The yield of product was 43%. ^{1}H NMR (DMSO-d_6), δ (ppm): 12.35 (br, 1H, 6-OH), 8.26 (s, 1H, 2-H), 8.06 (s, 1H, 8-H), 5.89 (d, 1H, J = 4.9 Hz, 1′-H), 5.58 (m, 2H, 2′-OH, 3′-OH), 5.34 (m, 4H, 2-CH=CH–), 4.54 (t, 1H, J = 4.9 Hz, 3′-H), 4.29 (m, 1H, 2′-H), 4.18 (m, 2H, 4′-H, 5′-H), 4.08 (t, 1H, J = 4.5 Hz, 5′-H), 2.73 (m, 2H, $=CH-CH_2-CH=$), 2.29 (t, 2H, J = 7.1 Hz, $-CH_2CO$), 2.18 (m, 2H, $-CH_2$), 1.98 (m, 4H, $-CH_2-$), 1.48 (m, 2H, $-CH_2-$), 1.23 (m, 14H, 7–CH_2-), 0.85 (t, 3H, J = 6.2 Hz, $-CH_3$).

Table 1
Enzyme screen for inosine esters synthesis

Enzyme	Conversion (%)[a,b]
Control, no enzyme	0
Alkaline protease from *Bacillus subtilis*	17
Lipozyme® immobilized lipase from *Mucor miehei*	65
Lipase from porcine pancreas	7
Lipase from *Candida rugose*	43
Lipase from *Mucor javanicus*	31
Lipase from *Candida cylindracea*	25

[a] Experimental conditions: 0.04 mmol inosine, 0.16 mmol vinyl laurate, 10 mg/ml enzyme, 1 ml acetone, 50 °C, 24 h.
[b] Conversion was determined by HPLC.

3. Results and discussion

3.1. Effect of enzymes

One of the most important parameters for enzyme-catalyzed reactions is the selection of enzyme sources. Six commercially available enzymes were tested for the transesterification of inosine with vinyl laurate in anhydrous acetone at 50 °C. The results were compared and listed in Table 1. From Table 1, it clearly showed that no transesterification occurred without enzyme participation. Lipozyme® exhibited its advantage to catalyze the reaction, while the lipase from porcine pancreas had the lowest catalysis activity.

3.2. Effect of organic solvents

Different solvents, listed in Table 2, were tested for the transesterification of inosine with vinyl laurate by Lipozyme® at 50 °C. The low esterification degrees were observed when 2-methyl-2-propanol or pyridine was used as solvent, while acetonitrile, dioxane and acetone exhibited the high conversion. Concerning the lower toxicity and

Table 2
Effect of solvent on the transesterification of inosine and vinyl laurate

Solvent	log P[a]	Conversion (%)[b]
DMF	−1.0	11
Dioxane	−0.5	68
Acetonitrile	−0.39	72
Acetone	−0.23	64
THF	0.46	53
Dichloromethane	0.6	28
Pyridine	0.65	9
2-Methyl-2-propanol	0.79	6
Toluene	2.6	0
Hexane	3.9	22

[a] Laane et al. [16].
[b] Experimental conditions: 0.04 mmol inosine, 0.16 mmol vinyl laurate, 10 mg/ml Lipozyme®, 1 ml solvent, 50 °C 24 h.

easier processing, acetone was chosen as reaction media for this transesterification. Ideally, a good solvent for biocatalysis should not only maintain the enzyme activity, but also dissolve substrates as well. Hydrophobilc solvents such as hexane were normally used in enzyme-catalyzed reaction [14,15]. But to this transesterification reaction, nonpolar solvents were unfavorable due to inosine solubility.

3.3. Effect of acylating agents

The acylating agents also affect both the catalytic efficiency and the regioselectivity of enzyme. Transesterification of inosine with different vinyl fatty acid ester were performed at 50 °C in acetone for 24 h catalyzed by Lipozyme® (Fig. 1). The result showed that among the six linear chain saturated vinyl fatty acid esters, the vinyl ester with carbon length of C8–C18 had nearly the same esterification degree (about 65%), and the highest yield was obtained in the case of vinyl caprylate. In contrast with saturated fatty acids, the esterification degrees of unsaturated fatty acids such as vinyl oleate (45%) and vinyl linoleate (53%) were lower.

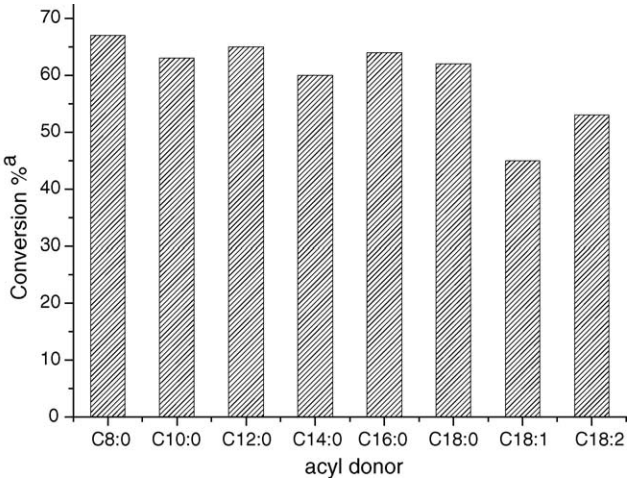

Fig. 1. Effect of chain length of acyl donor on the Lipozyme® catalyzed transesterification of inosine. Experimental conditions: 0.04 mmol inosine, 0.16 mmol acyl donor, 10 mg/ml Lipozyme®, 1 ml acetone, 50 °C, 24 h.

3.4. Enzymatic synthesis of 5′-O-fatty acid inosine esters

Transesterification of inosine with different vinyl fatty acid esters (C8–18) catalyzed by immobilized lipase from *M. miehei* in acetone was shown in Scheme 1. Only one spot was detected on TLC. The products were purified by silica gel chromatography and analyzed by ^{13}C NMR spectrometry to identify the position esterified, as shown in Table 3. According to the general strategy described by Yoshimoto et al. [17], acylation of a hydroxyl group will lead the *O*-acylated carbon (*CH_2OCOR) downfield, while the adjacent carbon (*CCH_2OCOR) upfield in ^{13}C NMR. Analysis of ^{13}C NMR spectra of all the products revealed that the products (**3a**–**h**) were substituted at the primary hydroxyl of inosine.

Table 3
Chemical shifts of ^{13}C NMR (DMSO-d_6) of inosine and inosine esters

Carbon	Inosine	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	**3g**	**3h**
2	145.8	146.48	146.34	146.35	146.41	146.36	146.30	146.35	146.35
4	148.1	148.79	148.65	148.66	148.69	148.66	148.63	148.66	148.67
5	124.3	125.10	124.97	124.98	124.99	124.98	124.98	124.98	125.00
6	156.5	157.12	156.98	157.00	157.05	157.00	156.96	157.00	157.01
8	138.7	139.36	139.21	139.21	139.26	139.23	139.16	139.22	139.23
1′	87.36	88.31	88.18	88.19	88.19	88.18	88.21	88.20	88.21
2′	70.22	70.80	70.66	70.67	70.69	70.64	70.67	70.66	70.69
3′	74.03	73.93	73.81	73.82	73.84	73.82	73.82	73.83	73.83
4′	85.53	82.26	82.13	82.13	82.15	82.13	82.12	82.13	82.15
5′	61.19	64.22	64.09	64.11	64.14	64.12	64.10	64.15	64.15
C=O		173.35	173.22	173.22	173.27	173.23	173.16	173.19	173.20
$-CH_2$		33.91	33.78	33.79	33.80	33.78	34.14	33.77	34.15
		31.66	31.69	31.75	31.79	31.76	33.77	31.74	33.78
		28.92	29.26	29.43	29.54	29.50	31.73	29.55	29.56
		28.89	29.10	29.43	29.51	29.48	29.46	29.50	29.51
		24.98	29.06	29.32	29.46	29.43	29.46	29.43	29.43
		22.58	28.83	29.15	29.36	29.33	29.40	29.29	29.30
			24.84	29.12	29.21	29.17	29.13	29.14	29.18
			22.52	28.85	29.16	29.14	28.85	28.94	28.99
				24.85	28.88	28.86	28.85	28.86	27.06
				22.55	24.88	24.96	24.83	27.02	25.68
					22.95	24.86	24.64	24.84	24.97
						22.56	22.52	22.42	22.42
$-CH_3$		14.48	14.39	14.41	14.46	14.43	14.36	14.38	14.37
-CH=CH-								130.18	130.19
								130.08	130.09
									128.22
									128.13

As listed in Table 3, the signals for carbon number 5′ shifted downfield from 61.2 to 64.2 ppm, and the signals for carbon number 4′ shifted upfield from 85.5 to 82.2 ppm. Therefore, the esterification of inosine catalyzed by the Lipozyme® inevitably occurred at primary hydroxyl. The results showed that Lipozyme® catalyzed a good regioselective modification of inosine similar to the previous reports on guaifenesin [18].

4. Conclusion

In conclusion, a facile route to prepare inosine esters bearing with a long chain acyl group on the primary hydroxyl position was developed in this paper. Different reaction conditions were examined and the best one was carried out in acetone at 50 °C under Lipozyme® catalyst. These 5′-*O*-acyl derivatives of inosine obtained are more lipophilic than the parent inosine and the potential usage of inosine esters are under investigation.

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