Pergamon

Tetrahedron: Asymmetry, Vol. 7, No. 11, pp. 3063-3066, 1996

0957-4166/96 $15.00 + 0.00

PII: S0957-4166(96)00398-9

Asymmetric Acylation with Polyethylene Glycol-Modified *Candida cylindracea* Lipase in Homogeneous Organic Media

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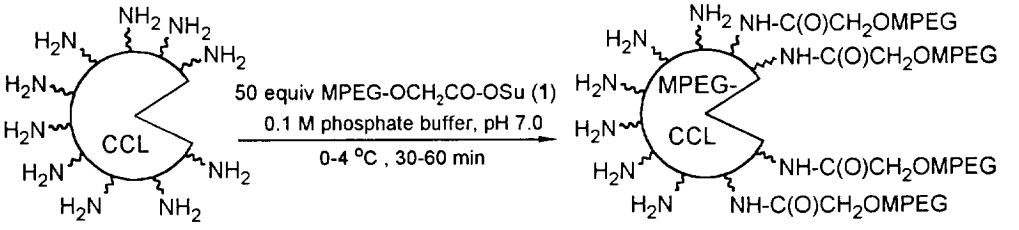
Abstract: MPEG-CCL was prepared by acylation of CCL with MPEG-OCH_2CO-OSu and characterized by FPLC and PAGE. MPEG-CCL is soluble in benzene and it retains 31% of the activity of CCL. In benzene MPEG-CCL has a significantly higher activity than CCL. The enantioselectivities of CCL- and MPEG-CCL-catalyzed acylations are similar. MPEG-CCL-catalyzed acylations with isopropenyl methoxyacetate and hydrolyses of methoxyacetates have especially high rates. Copyright © 1996 Elsevier Science Ltd

Catalysis by enzymes in organic media of low water content is an area currently receiving much attention.[1] Especially useful for synthetic purposes is the asymmetric acylation of racemic and prochiral alcohols catalyzed by lipases.[1b] Because of the insolubility of lipases in hydrophobic organic solvents, such acylations proceed under heterogeneous reaction conditions. It has been observed that lipases generally not only have a diminished activity in organic media but also different selectivities.[1b] Enzymes can frequently be made soluble in organic solvents while retaining their activity by the covalent attachment of amphiphilic polyethylene glycol monomethylether molecules (MPEG) to their lysine amino groups.[2] Recently we have prepared MPEG-modified pig liver esterase and studied its ability to catalyze the asymmetric acylation of diols in homogeneous organic solution.[3] We were now interested to see if there is a basic difference between lipase-catalyzed asymmetric acylations in heterogeneous and in homogeneous organic media. For this study we chose *Candida cylindracea* lipase (CCL; also named *Candida rugosa* lipase) which is a versatile hydrolase for asymmetric synthesis.[1,4] Several groups have described the modification of CCL either with cyanuric chloride activated MPEG or with MPEG-O-CO-OC_6H_4-NO_2 and studied the MPEG-CCL-catalyzed hydrolysis and acylation of achiral esters and alcohols, respectively.[5,6] As compared to CCL, MPEG-CCL showed a higher activity in benzene but a greatly reduced activity in water. Since CCL is a sensitive enzyme in aqueous solution,[7] we used for its pegylation MPEG-OCH_2CO-OSu (**1**) which is the most reactive pegylation reagent under neutral conditions.[2] Ester **1** was synthesized by carboxymethylation of MPEG-5000 with ethyl bromoacetate, saponification of MPEG-O-CH_2COOEt and subsequent treatment of thus obtained MPEG-O-CH_2COOH with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide.[8]

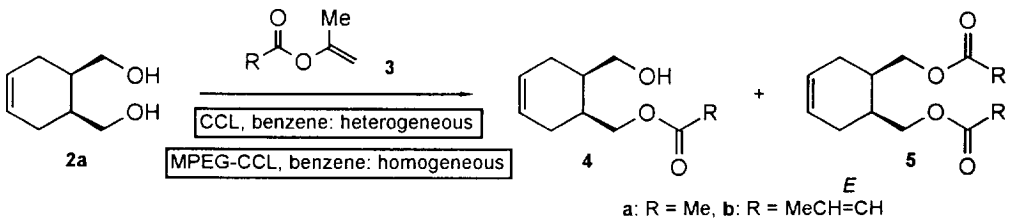


The contamination of commercial MPEG-5000 with bifunctional polyethylene glycol (PEG-10000)[9-11] was removed by repeated fractional crystallization.[10,11] The purification as well as the carboxymethylation of MPEG-5000 were both followed by HPLC (TSK G 3000 PWXL type column), which allowed for a separation of MPEG-5000 and PEG-10000[10] as well as of MPEG-5000 and MPEG-OCH_2COOH. Reagent **1** made possible the modification of CCL at pH 7.0 within 1 h at 0-4 °C. The MPEG-CCL prepared in this study

differs from the previously synthesized one by having a rather stable amide instead of a carbamate or a triazine protein-polymer linkage. Separation of MPEG-CCL from MPEG-OCH_2COOH, which was formed by hydrolysis of **1** during the modification procedure, was not possible in spite of a repeated ultrafiltration through a PM 30 membrane. The thus obtained preparation of MPEG-CCL contained 3-4% of protein as determined by the bicinchoninic acid (BCA) test.[12] The degree of pegylation of MPEG-CCL was 47% as revealed by the fluorescamine method.[13] MPEG-CCL was further characterized by FPLC and electrophoresis. It could be demonstrated by SDS-PAGE that the MPEG-CCL preparation contained no native CCL (≤5%). Electrophoresis (SDS-PAGE, PAGIEF, Disk-PAGE) showed clear differences between MPEG-CCL and CCL. In all Coomassie blue stained gels, however, MPEG-CCL could not be detected. Only esterase-zymograms with α-naphthyl acetate as substrate[5b,c] enabled the detection of MPEG-CCL in case the electrophoresis was done under non-denaturing conditions (PAGIEF, Disk-PAGE). PAGIEF in combination with α-naphthyl acetate staining revealed the expected lower pI-value of MPEG-CCL (3.56-3.73) as compared to CCL (4.06 and 4.17). Native anionic Disk-PAGE[14] showed also noticeable differences between CCL and MPEG-CCL. The MPEG-CCL preparation retained 31% of the activity of CCL in aqueous solution as determined by the *p*-nitrophenyl butyrate test.[15]

Contrary to native CCL MPEG-CCL is apparently soluble in benzene. The activity of MPEG-CCL in benzene solution is significantly increased as compared to CCL. Because of the low asymmetric induction of its CCL-catalyzed acylation, the diol **2a** was chosen for the comparative study between CCL and MPEG-CCL..

Table 1. MPEG-CCL- and CCL-catalyzed acylation of the diol **2a** (1 mmol) with the acetates **3a,b** (5 mmol) in benzene (20 mL) at 25 °C.



acyldonor/lipase[a-c]	t (h)	rate[d]	conversion (%)[e] monoester	diester	ee (%)[f]
3a/CCL	638	0.087	**4a** (50)	**5a** (3)	21
3a/MPEG-CCL	70	0.69	**4a** (46)	**5a** (1)	23
3b/CCL	123	0.44	**4b** (38)	**5b** (8)	29
3b/MPEG-CCL	48	2.43	**4b** (81)	**5b** (18)	40

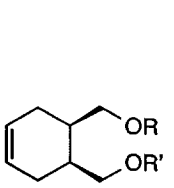
[a] 10 mg (180 U) of CCL was used which had a protein content of 40% and a specific activity of 45 U/mg protein. [b] CCL was a semi-purified, completely water soluble form of lipase CCL-VII (Sigma). Water insoluble residues were removed by centrifugation and low molecular-weight byproducts by repeated ultrafiltration with 0.1 M phosphate buffer of pH 7.0 through a PM 30 membrane at 5 °C. [c] 10 mg of MPEG-CCL was used which had a protein content of 3-4% and a specific activity of 14 U/mg protein. [d] In μmol/h·mg enzyme. [e] Determined by GC. [f] Determined by 500 MHz ^{1}H NMR spectroscopy of the (S)-MTPA-esters of **4a** ($CDCl_3$) and **4b** (C_6D_6).

For the CCL- and MPEG-CCL-catalyzed acylations of the diol **2a** isopropenyl acetate (**3a**) and isopropenyl-*E*-crotonate (**3b**) (Table 1) were used as acylation reagents. A 77- and 55-fold higher specific rate (U/mg protein), respectively, of the acylation catalyzed by MPEG-CCL was observed. The enantioselectivities of the acylations catalyzed by CCL and by MPEG-CCL are almost the same. These results are somewhat surprising since a noticeable change in the enantioselectivity of acylation was observed upon the non-covalent[4b,d] and covalent.[4e,f]

CCL-catalyzed acylations of alcohols proceed with exceptional high rates even at low temperatures in case trichloroethyl methoxyacetate is used as the acylating reagent in ether.[16] We therefore prepared isopropenyl

methoxyacetate (**3c**)[17] which should allow for an essentially irreversible acylation. The MPEG-CCL-catalyzed acylation of the diols **2a** and **6a-8a** with **3c** was investigated at room temperature in homogeneous benzene solution (Table 2). In control experiments the hydrolysis of the corresponding methoxyacetates **2c** and **6c-8c** in aqueous buffer solution was studied, too.

Table 2. MPEG-CCL-catalyzed acylation of the diols **2a** and **6a-8a** in benzene with the acetate **3c** at room temperature and CCL-catalyzed hydrolysis of the esters **2c** and **6c-8c** in 0.01 M TRIS-HCl buffer at pH 7.0 and 36-40 °C.



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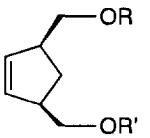

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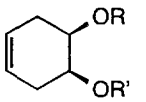

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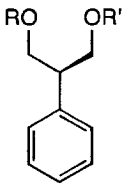

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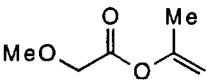

3c

a: R = R'= H, **b**: R = H, R'= $COCH_2OMe$, **c**: R = R'= $COCH_2OMe$

substrate (mmol)/acyl-donor (mmol)/enzyme (mg)	t (min)	rate[a,b]	conversion (%)[c] monoester	diester/diol	yield (%)	monoester $[\alpha]_D^{20}$ [d]	ee (%)[e]
2a (0.50)/**3c** (2.50)/ MPEG-CCL (10)	95	31	**2b** (78)	**2c** (11)	54	+0.6 (*c* 2.9)	5
2c (1.40)/-/CCL (35)[f]	27	81	*ent*-**2b** (68)	**2a** (7)	61	-3.0 (*c* 9.2)	23
6a (1.12)/**3c** (5.50)/ MPEG-CCL (10)	85	27	**6b** (38)	**6c** (1)	40	-12.2 (*c* 3.8)	62
6c (1.93)/-/CCL (43)[f]	10	146	*ent*-**6b** (46)	**6a** (1)	51	+9.2 (*c* 9.8)	43
7a (0.88)/**3c** (4.40)/ MPEG-CCL (10)	270	13	**7b** (62)	**7c** (2)	54	-0.6 (*c* 4.4)	2[g]
7c (1.88)/-/CCL(43)[f]	26	52	*ent*-**7b** (49)	**7a** (1)	30	+3.1 (*c* 5.0)	24[g]
8a (0.66)/**3c** (3.30)/ MPEG-CCL (10)	175	18	**8b** (67)	**8c** (7)	47	-2.9 (*c* 3.5)	23
8c (1.47)/-/CCL (55)[f]	5	151	*ent*-**8b** (60)	**8a** (3)	60	+1.0 (*c* 10)	13

[a] No reaction occurred in the absence of the enzyme. [b] Calculated as total rate of acylation (μmol/h·mg enzyme) by assuming a constant rate until the termination of the reaction. [c] Determined by GC. [d] In $CHCl_3$. [e] Determined by 500 MHz ^1H NMR spectroscopy of the (S)-MTPA-esters in C_6D_6. [f] Crude CCL from Sigma (CCL-VII) was used. [g] Determined by GC on a permethylated β-cyclodextrin column.

In the case of the MPEG-CCL-catalyzed acylation of the diol **2a** with the methoxyacetate **3c** a more then tenfold rate acceleration as compared to isopropenyl butyrate, valerate, caproate and crotonate was observed.[18] The rate of hydrolysis of the methoxyacetates **2c**, **6c**, **7c** and **8c** was also significantly enhanced. As shown in Table 2, the use of this acyl moiety allows the CCL-catalyzed hydrolysis of 2 mmol substrate to proceed within a few minutes even in the case of an ester of a secondary alcohol. The ee-values of **2b**, **6b** and **8b** are comparable to those of the corresponding monoacetates obtained either by CCL-catalyzed acylation of the above diols (vinyl acetate or ethyl acetate) or hydrolysis of the corresponding diacetates.[19-21]

In conclusion, there seems to be no significant difference in the enantioselectivity of the CCL-catalyzed acylation in heterogeneous and in homogeneous organic media. The application of MPEG-CCL instead of CCL in combination with isopropenyl methoxyacetate could be useful in cases were the acylation of a racemic alcohol or a prochiral diol with the native enzyme under heterogeneous reaction conditions is highly selective but too slow. MPEG-CCL can be recovered by ultrafiltration if desired.

Acknowledgment. Financial support of this work by the Deutsche Forschungsgemeinschaft (SFB 380) is gratefully acknowledged.

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20. The absolute configuration of **4b**, **6b** and **8b** was assigned by correlation with the corresponding acetates.[19]
21. Satisfactory spectral and analytical data were obtained for all new compounds.

(*Received in UK* 9 *August* 1996; *accepted* 18 *September* 1996)