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Enzymatic Kinetic Separation of Stereoisomeric Macrocyclic Lactone Derivatives, 7α,β-O-Acyl *trans*-Zearalenols and 7α,β-O-Acyl Zearanols

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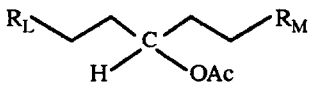
Key words: Lipases, diastereoselectivity and site-selectivity of hydrolysis, 7α,β-O-acyl derivatives of resorcylic acid macrocyclic lactones

Abstract: Diastereomeric mixtures of resorcylic acid macrocyclic lactones, 7α,β-O-acyl zearalenols (**1,2** and **3,4**, full name: *trans*-3,4,5,6,9,10-octahydro-14,16-dihydroxy-7-acyloxy-3-methyl 1H-2-benzoxacyclotetradecin-1-ones (3S,7S or 3S,7R)), and 7α,β-acyl zearanols (**5,6** and **7,8**, full name: 3,4,5,6,9,10,11,12-decahydro-14,16-dihydroxy-7-acyloxy-3-methyl-1H-2-benzoxacyclotetradecin-1-ones (3S,7S or 3S,7R)) are hydrolyzed by lipases. Kinetic separation affords 7β-alcohols with complete site-selectivity and nearly complete diastereoselectivity. Among lipases examined, those from *Pseudomonas sp.*, *Pseudomonas fluorescens* and *Candida cylindracea* exhibit the broadest substrate specificity. In all cases 7β-alcohols are major products, diastereomeric excess (d.e. in %, determined by HPLC) approaches 100% for **1,2**, 80% for **3,4**, and varies between 10% and 90% for **5,6** and **7,8**, respectively. Faster and less stereoselective hydrolysis is observed for 7α,β-O-chloroacetyl derivatives **3,4** and **7,8** as compared with acetyl derivatives **1,2** and **5,6**. The effect of ionic strength and of organic cosolvent on the rate and selectivity is studied. Explanation of stereoselectivity of this enzymatic reaction is based on the conformational properties of macrocyclic ring around the chiral acyloxymethine center C(7). Perturbation by the local helical arrangement of the two trimethylenic side-chains attached to this center renders 7β-O-acyl diastereomers much more reactive counterparts.

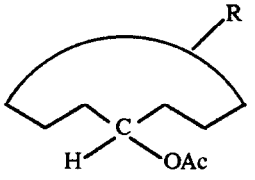
INTRODUCTION

Lipase catalyzed kinetic resolution by enantioselective hydrolysis of racemic acyl derivatives of *sec* alcohols is widely studied on the laboratory scale,[1] and some pilot-plant experiments are also reported.[2] Generally, high enantioselectivity was reached[3-8] when substrates possess on the chiral center largely different groups R_L, R_M, as first reported by Ziffer et al.[3,4] for the whole cells of *Rhizopus nigricans*, and later for lipolytic enzymes isolated from various microorganisms.[5-8] Less satisfactory resolution is obtained for aliphatic *sec* alcohols possessing conformationally free alkyl groups with similar sterical requirements (**I**).[9,10] This situation could drastically change in cyclic carbinols, however, since rotation of the two side chains on the acyloxymethine center is restrected by their connection into macrocyclic structure (**II**). High stereoselectivity of lipase catalyzed hydrolysis[12,13] and esterification[13a,13b] of the substrates with small rings, substituted vicinally to the chiral hydroxymethine center, suggests such possibility.

Certain conformational rigidity is present in the medium and large rings. Conformational analysis of 14-membered lactones is recently reported,[14] as well as on profound effects of conformational control on the reactivity of the medium and large rings.[15] Chiral center in compounds **II** bears *sec* acyloxy group and can be available to lipases preferably from one (dia)stereoface. We investigated this concept on one example of commercial importance, *i.e.* on the stereoselective lipase catalyzed hydrolysis of 7α,β-O-acyl derivatives of macrocyclic lactones **1-8**. Since it is observed that lipases open some small ring lactones,[16,17] it was also of interest to prove their site-selectivity with O-acyl substituted larger-ring lactones.

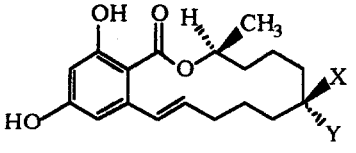


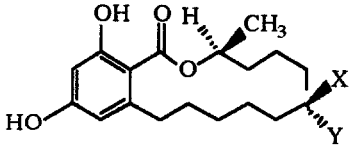
I



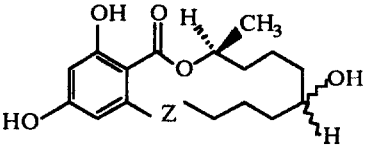
II

Stereochemically pure 7α-alcohol **11** is well known growth promoting factor zearanol.[18,19] This compound is obtained by hydrogenation of both *trans*-double bond and ketone carbonyl group in the naturaly occuring resorcylic macrocyclic lactone zearalenone (**13**), and presently is available in stereochemically pure form only by tedious separation of diastereomers **11,12**[20] or their acyl derivatives **5,6**[21,22] *via* repeated crystallization, or by kilo-prep HPLC separation of **11,12**.[23]

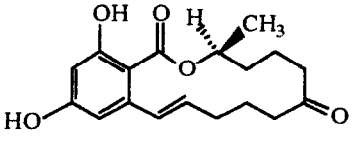




	X	Y		
1	H	$OCOCH_3$	**5**	(7α)
2	$OCOCH_3$	H	**6**	(7β)
3	H	$OCOCH_2Cl$	**7**	(7α)
4	$OCOCH_2Cl$	H	**8**	(7β)



	Z	
9	CH= CH	7α
10	CH= CH	7β
11	CH_2-CH_2	7α
12	CH_2-CH_2	7β



Zearalenon, **13**

RESULTS AND DISCUSSION

In the first experiments a series of commercially available lipases was examined with diastereomeric mixtures **1,2** and **5,6**, Table 1. Beside those listed in the Table, lipases from *Humicola lanuginosa, Penicillium camambertii, Geotrichum candidum and Candida lipolytica* were tested, and proved virtually inactive.

Lipases from *Pseudomonas sp.* and *Pseudomonas fluorescens* selectively hydrolyse 7β-diastereomers **2** and **6**, with known 3S,7S absolute configuration.[24,25] The unsaturated substrate **2** was hydrolyzed faster than **6**, with complete diastereoselectivity. Lipase from *Candida cylindracea* was less active with diastereomeric mixture **1,2**, whereas lipases from *Aspergillus niger* and *Rhyzopus oryzea* exhibited very low activity, all retained complete β-diastereoselectivity, however. Lipases from *Pseudomonas sp.* and *Pseudomonas fluorescens* slowly deacylate a mixture of saturated congeners **5,6**, with *ca.* 80% diastereoselectivity. The progress curves for hydrolysis of *ca.* 1:1 mixtures **1,2** with five active lipases are presented in Fig. 1., and reveal varying conversion rates of 7β-diastereomer **2**.

Table 1: Hydrolysis of 7α,β-O-acetyl-zearalenols (**1,2**) and 7α,β-O-acetyl zearanols (**5,6**) with lipases.

Substrate	Lipase	t/h	T/°C	Conversion, %	Product configuration	d.e., %
Diastereomeric mixture **1,2**	*Pseudomonas species*	72	45	48.1	**10** 3S,7S (7β)	100
	Candida cylindracea	96	37	21.3		100
	Aspergillus niger	76	R.t.	3.4		100
	Rhizopus oryzae	96	R.t.	5.4		100
	Pseudomonas fluorescens	48	40	49.4		100
Diastereomeric mixture **5,6**	*Pseudomonas species*	94	45	17.5	**12** 3S,7S (7β)	80.1
	Pseudomonas fluorescens	96	40	26.9		93.4

On the completed screening, lipase from *Pseudomonas sp.* was used throughout this study. To establish eventual inhibitory effect of one diastereomer over the other, **1** and **2** were separately hydrolyzed under identical conditions. It was possible to confirm complete inertness of 7α-isomer **1**, which remained unattacked after 94 hours, wheres 7β-isomer **2** was converted to 90%, Fig. 2. This result is analogous to that obtained with a mixture **1,2**, and eliminates mutual inhibitory effect.

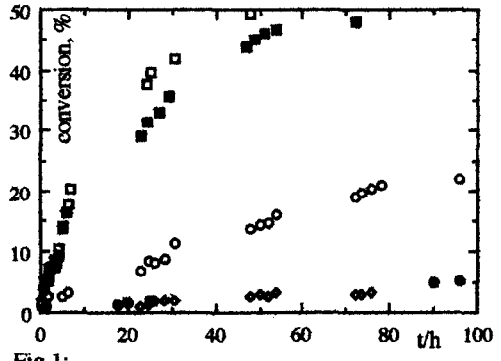


Fig.1:
Progress curve of hydrolysis of **1,2** with lipases from *Candida cylindracea* (○), *Aspergillus niger* (◆), *Pseudomonas sp.* (■), *Rhizopus oryzae* (●) and *Pseudomonas fluorescens* (□).

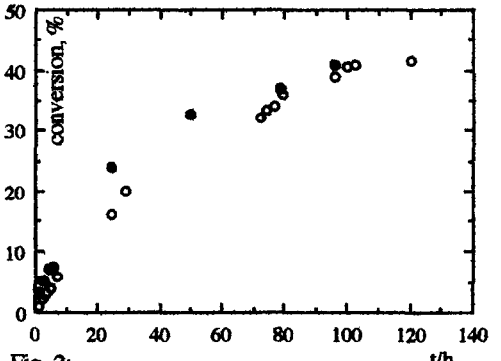


Fig. 2:
Progress curve of hydrolysis of diastereomeric mixture of **1,2** (●), as compared with hydrolysis of **2** (○), with *Pseudomonas sp.* lipase.

The effect of buffer concentration on *Pseudomonas sp.* catalyzed hydrolysis was examined with **1,2**, since in some cases notable effect of ionic strength on the rate and selectivity of lipases was reported.[26,27] Relative rates and conversions of **1,2** remained unchanged in 0.1-1.0 M K-phosphate buffer.

It is also repeatedly noticed[28,29] that limited quantities of organic solvents miscible with water enhance the rate. With **1,2** the rate of hydrolysis was notably higher with 20% methanol in 0.1 M buffer, while maintaining high diastereoselectivity. Addition of 50% methanol to 0.1 M buffer reverses the progress curve back to that observed for aqueous solution, Fig. 3.

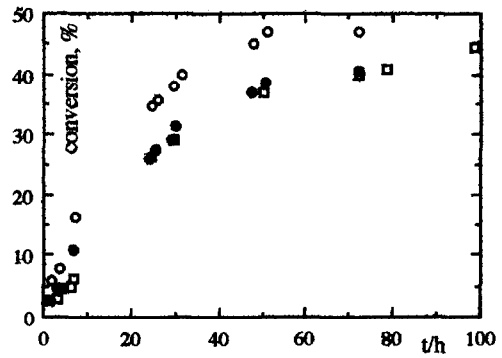


Fig. 3:
Effect of added methanol to 0.1M buffer on hydrolysis of **1,2** with *Pseudomonas sp.* lipase (□ 0 %, ○ 20 %, ● 50 % MeOH).

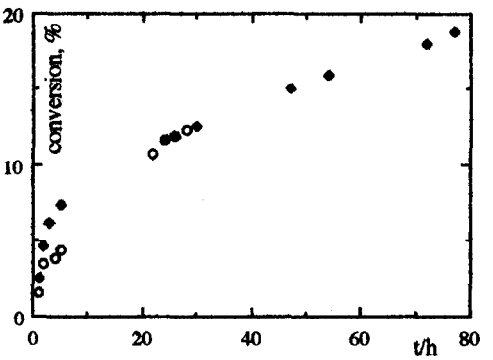


Fig. 4:
Hydrolysis of **5,6** with *Pseudomonas sp.* (○) and *Pseudomonas fluorescens* lipase (◆).

Screening of the pair of 11,12-unsaturated diastereomers **5,6** with the aforementioned lipases revealed lipases from *Pseudomonas sp.* and *Pseudomonas fluorescens* as the only active ones, Table 1. Progress curves for conversion of this diastereomeric mixture, Fig. 4., shows slower conversion as compared to **1,2**.

Seebach[30] and Sih[31] demonstrated that the structure of acyl group effects the rate and selectivity of lipase catalyzed hydrolysis of racemic O-acyl derivatives. Hydrolysis rate seems to be enhanced when an electron-withdrowing group (Cl, CN) is introduced into methyl group of the acetyl residue. To check this phenomenon on macrocyclic substrates, diastereomeric chloroacetoxy derivatives **3,4** and **7,8** were prepared and enzymaticaly hydrolyzed. Compounds **3,4** and **7,8** undergo faster hydrolysis than **1,2** and **5,6**, respectively. For **3,4** d.e. remains *ca* 80% after prolonged reaction time, whereas for **7,8** two active lipases exhibited low to medium diastereoselectivity (d.e.~10-65 %), Table 2.

Table 2: Hydrolysis of 7α,β-O-chloroacetyl zearalenols (**3,4**) and 7α,β-O-chloroacetyl zearanols (**7,8**) with lipases.

Substrate	Lipase	t/h	T/ °C	Conversion, %	Product configuration	d.e., %
Diastereomeric mixture **3,4**	*Pseudomonas species*	7	40	25.2	**10** 3S,7S (β)	100
		48		57.0		73.6
	Candida cylindracea	22	R.t.	13.3		100
		28		17.0		81.7
	Pseudomonas fluorescens	8	40	25.2		100
		28		47.1		79.9
Diastereomeric mixture **7,8**	*Pseudomonas species*	2.5	40	20.2	**12** 3S,7S (β)	64.7
		24		53.7		34.8
		44		85.7		10.8
	Pseudomonas fluorescens	2.5	40	18.1		59.9
		24		54.4		28.5
		48		81.9		15.7

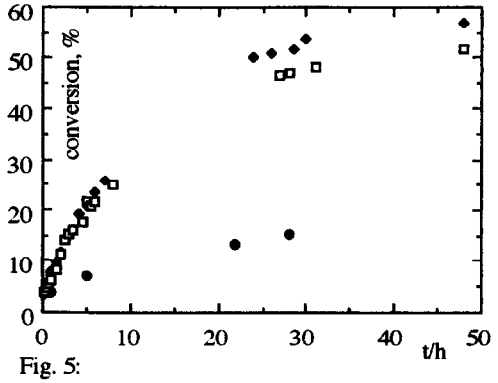


Fig. 5:
Progress curves for hydrolysis of **3,4** with lipase from *Pseudomonas sp.* (♦), *Pseudomonas fluorescens* (□), and *Candida cylindracea* (●).

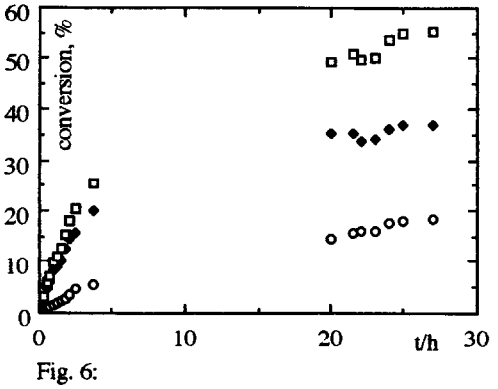
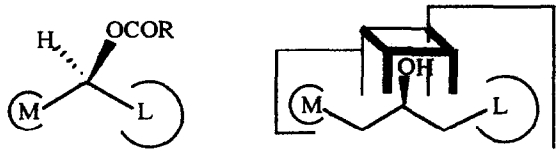


Fig. 6:
Progress curves for conversion of single diastereomers (○, **7**), (♦, **8**) in the mixture **7,8** (□), and their summation in hydrolysis with *Pseudomonas sp.* lipase.

Lipase from *Pseudomonas sp.* reveals nearly complete diastereoselectivity in the first period (7-8 h) of hydrolysis of **3,4**, which later falls down to *ca* 80%, Fig.5. Hydrolysis of **7,8** proceeded with 60-65% d.e. in the first 2.5 hours, after 44-48 hours d.e. fell down to *ca.* 10-16%, Table 2. and Fig. 6. This Figure also shows two different regions of the progress curves; noticable difference of the relative rates for **7** and **8** in the innitial period, and nearly constant ratio of these rates in the final period of hydrolysis.

In conclusion we can state that among the enzymes tested, lipase from *Pseudomonas sp.* and *Pseudomonas fluorescens* exhibited nearly complete diastereoselectivity (d.e. 80-100 %) in hydrolysis of diastereomeric mixtures of 7α,β-O-acyl derivatives of zearalenol (**1-4**) and 10-80 % d.e. for analogous zearanol derivatives **5-8**. At optimal pH, temperature, and solvent mixture complete kinetic separation of **1** from **2** and **3** from **4** is achieved, rendering enzymatic process promissing also for the large scale separation of such biotechnological products. Moreover, 7β-diastereoselectivity opens the possibility of chemoenzymatic transformation of diastereomeric mixture of 7α,β-isomers into commercialy important 7α-alcohols. This consists of acylation of the crude mixture of the products of enzymatic hydrolysis by the strong acids, and successive hydrolysis of the mixture of esters under such conditions that 7α–O-acyl derivatives hydrolyse with retention, and 7β-O-acyl derivatives with inversion of configuration. This overall processs is the subject of our pending patent.[32]

To account for selective hydrolysis of 7β-O-acyl derivatives **2** and **6**, we should first recall two simple models recently proposed for predicting which substrate will be resolved efectively by lipases,[7,13b] Fig 7. Both are



a **b**

Fig. 7: **a.** An extension of Prelog's rule to hydrolases, more reactive enantiomer presented (according to ref. 7), **b.** representation of the enzyme active site with "canopy" for recognition of the hydroxyl functionality of the substrate (according to ref.13b).

nearly two-dimensional, and does not take into account conformational characteristics of the substrate. The latter however, seem to be of decisive importance in explanation of the lipase stereoselectivity with macrocyclic O-acyl derivatives.

Conformational properties of specific 14-membered macrocyclic lactones **1-13** are known. Structure and conformation are known for the solid state of 7α-O-acetyl derivatives **1**[24] and **5**,[25] whereas solution conformation of the series of 7α- and 7β diastereomers and the parent ketones is studied by NMR[33] and CD.[25,34] Our CD studies reveal substantially different solution conformations for 7α-derivatives **1,5,9**, and **11**, as compared to 7β-isomers **2,6,10** and **12**. The former exhibit much stronger and well defined Cotton effects. As X-ray data for two 7α-O-acetyl derivatives, **1**(Fig 8a.) and **5**, reveal, there is specific helical arrangement of the two trimethylenic side chains on the hydroxymethine chiral center C(7), Fig. 8b.

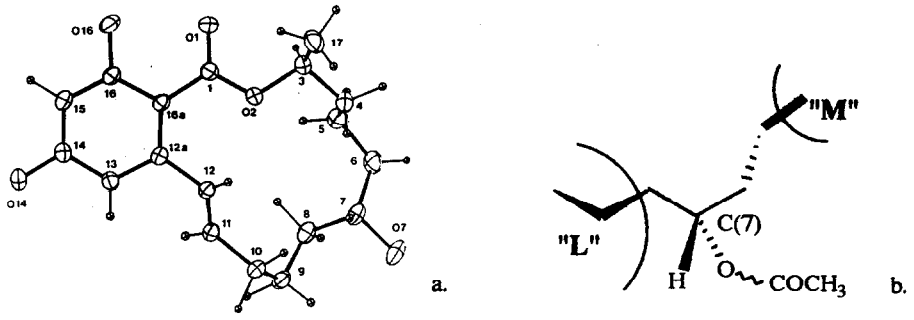


Fig.8. **a**. Ring conformation of **1** in the solid state (according to ref. 24), **b**. helical arrangement of trimethylenic chain, the left-sided, "bulky, L" chain effects the approach of nucleophile.

Left-hand side of this chain is protracted outside of the vertical plane which bisects C(6) and C(8), and therefore strongly perturbes the nucleophile on its approach to the acyloxy group on C(7), whereas the right-hand chain is placed in the rear side of the same plain, and leaves open space to the approach of the nucleophilic group on the active site of the enzyme. One can see that sterical requirements in 7α-diastereomer correspond to those in the non-reactive open chain substrates outlined in Fig. 7.

Our CD studies[25,34] revealed that 7β-isomers **2,6,10** and **12** adopt a quite different conformation, which seems not so well defined as for 7α-isomers, and rises less intensive Cotton effects than the latter. Since for 7β-isomers more energetically similar conformations at ambient temperature are excluded by determining the temperature dependent CD spectra, there seems to exist one conformation with less pronounced helicity and of the sign oposite to this for 7α-isomers.[34] This could explain high diastereoselectivity of lipases with macrocyclic derivatives.7β-Acetates **2,4,6**, and **8** seem to posses "correct" helicity around the chiral hydroxymethine center, and are therefore hydrolyzed preferentially. Somewhat lower 7β-diastereoselectivity, regularly achieved with 11,12-saturated congeners **5-8**, as compared with the unsaturated ones (**1-4**), Tables 1. and 2., presumably reflects less pronounced conformational difference between 7α- and 7β–isomers than in the latter. Solid state structure of **5**[25] reveals not so well defined helicity around the acetoxymethine center as compared to that in **1** (Fig 8.).

The above proposal seems to be supported by the recent finding concerning the active site of lipases.[35,36] X-Ray structure of the *Mucor mihei* lipase has been resolved, and reveals a trypsine-like Ser...His...Asp catalytic triade with an *active serine burried under short helical fragment* of a long surface loop.[35] The reported results indicate that the sence of the conformational helicity around the chiral center, bearing acetoxymethine group within conformationally well defined subunit of the substrate, can play decisive role in stereoselection by lipases. Other macrocyclic substrates with specifically designed conformational properties around the chiral center are needed for study of the herewith proposed matching between helicity around the chiral center of the substrate and that at the active site of lipases.

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EXPERIMENTAL

Column chromatography was performed on Silica Gel (Merck Kieselgel 60, 230-400 mesh). High pressure liquid chromatography was performed on a Hewlett Packard instrument Series 1050, with UV detector HP Series 1050 (λ 254 nm), using Waters Nova-Pak C18 (10 cm) reverse phase column. Hydrolysis was monitored, and d.e.'s determined by HPLC under following conditions: water-methanol-acetonitrile 40:30:30 as eluent, flow rate 1 ml/min, column temperature 21°C. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Jeol FX 90Q FT spectrometer; shifts are given in ppm downfield from TMS as an internal standard.

Preparation of the compounds 1-12: Diastereomeric mixtures of **1-4** were prepared by esterification of the corresponding 7-hydroxy compounds **9-12** (supplied by CRC, Chemical Research Company (UD), Italy), according to the literature method.[37,38]

Zearalenol 7α,β-chloroacetyl esters (3,4).- To a stirred solution of **9,10** (4.68 mmol) in 15 mL of acetone chloroacetyl chloride (5 mmol) was added in one portion at room temperature. Reaction mixture was warmed to 55-60 °C over 22 hours. After cooling to the room temperature excess of chloroacetyl chloride was evaporated and crude product was purified by column chromatography on silica gel (ethylacetate-dichloromethane 1:2 as eluent) to give 70% of **3,4**. Crude product was crystallized from toluene to give chemicaly pure material, m.p. 142-144°C. HPLC anal. (H_2O-MeOH-MeCN 40:30:30) 35.5% **3** and 64% **4**. ^{1}H-NMR: 1.345, 1.405 (dd, J=3.52Hz, 3H) overlapped with broad signal 1.64 (s, 12H), 2.18 (broad s, 2H), 4.06 (s, 2H), 5.37 (broad s, 2H), 5.91 (d, J 7.03 Hz, 1H, Ar-H), 6.41 (d, J 9.09 Hz, 1H, Ar-H), 11.35 (s, 1H, Ar-OH), 12.32 (s, 1H, Ar-OH). ^{13}C-NMR: 18.51, 18.74, 21.05, 21.39, 22.28, 22.57, 28.44, 28.89, 30.19, 30.93, 32.22, 32.85, 33.87, 34.43, 41.20, 72.53, 73.59, 73.69, 74.49, 102.27, 102.49, 103.61, 105.31, 107.96, 108.87, 131.77, 133.13, 142.55, 144.30, 160.28, 160.61, 163.24, 165.47.
Anal. Calcd. for $C_{20}H_{25}O_6Cl$: C, 60.53 %, H, 6.35 %, Cl 8.93 %. Found: C, 60.68 %, H, 6.60 %, Cl. 9.03 %.

Zearaenol-7α,β-O-chloroacetyl esters (7,8).- These compounds are prepared from **11,12** according to the above procedure. Crude product mixture was crystallized from diisopropyl ether to give 80% of pure **7,8**, m.p. 143-145°C. HPLC anal. (H_2O-MeOH-MeCN 40:30:30) 67% **7** and 33% **8**. ^{1}H-NMR: 1.37 9d, J 6.11 Hz, 3H) overlapped with broad signal 1.56 (s, 16H), 3.28 (m, 1H), 4.05 (s, 2H), 5.13 (m, 1H), 6.27 (dd, J 16Hz, 2H, Ar-H), 11.63 (s, 1H, Ar-OH), 12.23 (2, 1H, Ar-OH). ^{13}C-NMR: 19.51, 20.12, 21.02, 21.29, 23.34, 26.55, 27.15, 27.76, 28.13, 30.84, 32.09, 34.36, 34.99, 35.43, 36.96, 41.04, 73.39, 73.82, 101.54, 104.89, 105.67, 110.53, 110.78, 148.50, 148.83, 160.57, 164.75, 165.82, 167.08, 167.63, 171.32, 171.81.
Anal. Calcd. for $C_{20}H_{27}O_6Cl$: C, 60.22 %, H, 6.82 %, Cl 8.89 %. Found: C, 60.42 %, H, 6.63 %, Cl. 8.82 %.

Lipase mediated resolution of 7α,β-O-acyl substrates 1-8.

Lipases from *Pseudomonas sp., Candida cylindracea, Aspergillus niger, Rhizopus oryzae, Candida lipolytica, Humicola lanuginosa, Penicillium camambertii* and *Geotrichum candidum*, obtained from Amano Co., as extracellular enzymes of unknown activity. *Pseudomonas fluorescens* lipase (activity 42 units per mg of solid) was purchased from Fluka Co.

Enzymatic hydrolysis was performed in a thermostated reactor (TTA 80, Radiometer-Copenhagen) connected to a titrator TTT 80 with an autobirete ABU 80. pH was kept constant with pH M 82 equiped with glass-calomel electrode.

General Procedure: A mixtures of the 7α,β-O-acyl substrates **1,2**, **3,4**, **5,6**, **7,8**, (20 mg of each *ca* 1:1 to 1:2 diastereomeric mixtures) and lipase (20 mg,, only 1/10, *ca.* 2 mg of *Pseudomonas fluorescens* lipase, were used) in 0.1 M phosphate buffer (pH 7.0, 12 ml) and methanol (3 ml) was stirred at optimal temperature for each enzyme, as indicated in the Tables 1. and 2. The pH was kept constant by controlled addition of 0.1M NaOH. Samples (200 µl) were taken at the time intervals indicated in the Figs. 1-8, methanol (1 ml) was added, resulting suspension was filtered through a millipore filter and 2 µL samples injected into HPLC. Respective alcohols and acetates can be separated on a preparative scale using silica gel column with ethylacetate-dichloromethan 1:2 as eluent. R_f(**1,2**) 0.80, R_f(**3,4**) 0.74, R_f(**5,6**) 0.78, R_f(**7,8**) 0.85, R_f(**9,10**) 0.50, R_f(**11,12**) 0.47.

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