The First Kinetic Enzymatic Resolution of Methyl Ester of C75

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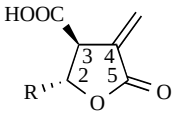
Abstract: Enantioselective hydrolysis of methyl ester of (±)-C75 was successfully accomplished by means of Acylase I from *Aspergillus* to afford (2*R*,3*S*)-(+)-C75 with 96% e.e. The unreacted methyl ester was recovered with >99% e.e. This latter compound was either chemically or enzymatically hydrolyzed to furnish (2*S*,3*R*)-(–)-C75 with >99% e.e.

Keywords: C75, enzymatic resolution, α-methylene-γ-butyrolactone, paraconic acid, Acylase I.

INTRODUCTION

Tetrahydro-4-methylene-2-octyl-5-oxo-3-furancarboxylic acid, designated as C75 [1] in the recent literature, is a synthetic compound [2]. It belongs to the class of paraconic acids which are characterized by the γ-lactone moiety, an alkyl chain at C-2, a carboxylic group at C-3, and a methyl or a methylene group at C-4 [3]. Paraconic acids having a methylene group at C-4 are in general biologically active natural compounds Fig. (**1**) [4-13].



(+)

1: R = *n*-C_3H_7
2: R = *n*-C_5H_{11}; Methylenolactocin
3: R = *n*-C_8H_{17}; C75
4: R = *n*-$C_{11}H_{23}$; Nephrosterinic acid
5: R = *n*-$C_{13}H_{27}$; Protolichesterinic acid

Fig. (1). Structure of paraconic acids **1-5**.

For example, (2*R*,3*S*)-(+)-(**1**), a toxin that induces formation of black spots on the peel of banana, was recently isolated from the plant pathogen *Lasiodiplodia theobromae* [4]. (–)-Methylenolactocin (**2**) isolated from the culture filtrate of *Penicillium sp.* [5] is active against some Gram-positive bacteria and it prolongs the life span of mice inoculated with Ehrlich carcinoma [6]. (+)-Nephrosterinic acid (**4**) was isolated from lichen *Nephromopsis endocrocea* [7] and (+)-protolichesterinic acid (**5**), isolated from various sources of *Cetraria* [8], from yellow *Acarospora* species of subgenus *Xantotallia* [9] and from lichens of *Parmelia* species [10], exhibiting *in vitro* anti-bacterial activity against *Helicobacter pylori* [11], there by inhibiting 5-lipoxygenase [12] and it also showed anti-proliferative effects on malignant cell-lines and mitogen stimulated lymphocytes *in vitro* [13].

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C75 is a fatty acid synthase (FAS) inhibitor and when administered in racemic form, it causes anorexia and reversible weight loss in rodents [14]. It also shows significant *in vivo* antitumor activity in human cancer cells [2a,15], suppresses DNA replication, induces apoptosis in tumor cell lines [16] and it is active against mycobacteria of the tuberculosis complex [17].

A considerable body of synthetic information exists in the literature to prepare paraconic acids having a methylene group at C-4 in both racemic and enantioselective version [3,18]. Among these methods, an efficient route was reported by Carlson and Oyler in 1976 [19]. In a slightly modified version [2,20] of their procedure, the dianion of 4-methoxybenzyl itaconate was condensed with aldehydes of various chain lengths to give in one step, after rapid exposure to strong acid, the desired α-methylene-γ-butyrolactone carboxylic acid. Both *trans*- and *cis*-isomer were generated by this method and these diastereomers could be separated by flash column chromatography. By this procedure, compounds (**1**) [20], (**2**) [20], (**3**) [2], (**4**) [19] and (**5**) [19] were synthesized in racemic form.

Here we report the enzymatic resolution of racemic methyl ester of C75 with Acylase I [21] as a preliminary investigation on the use of enzymatic hydrolysis for the obtainment of compounds (**1**)–(**5**) in enantiomerically pure form. For our present investigation, the methyl ester of C75 was synthesized in racemic form by exploiting a modified procedure with respect to that reported by Carlson and Oyler [19].

RESULTS AND DISCUSSION

Synthesis of the Methyl Ester of (±)-C75

The synthesis of racemic *trans* methyl ester of C75 (**12**) [17] (Scheme **1**) started with the regioselective ring-opening of itaconic anhydride (**6**) with methanol to give itaconic acid methyl hemiester **7** [22]. Treatment with lithium bis(trimethylsilyl)amide converted the hemiester into the corresponding ester enolate (**8**), which was reacted with nonanal. After acidification at –78 °C, a 3:2 mixture of the corresponding *syn* and *anti* hydroxy acids (**9**) and (**10**) was obtained. They were not isolated but only identified by ^{1}H NMR analysis of the crude reaction mixture. Their

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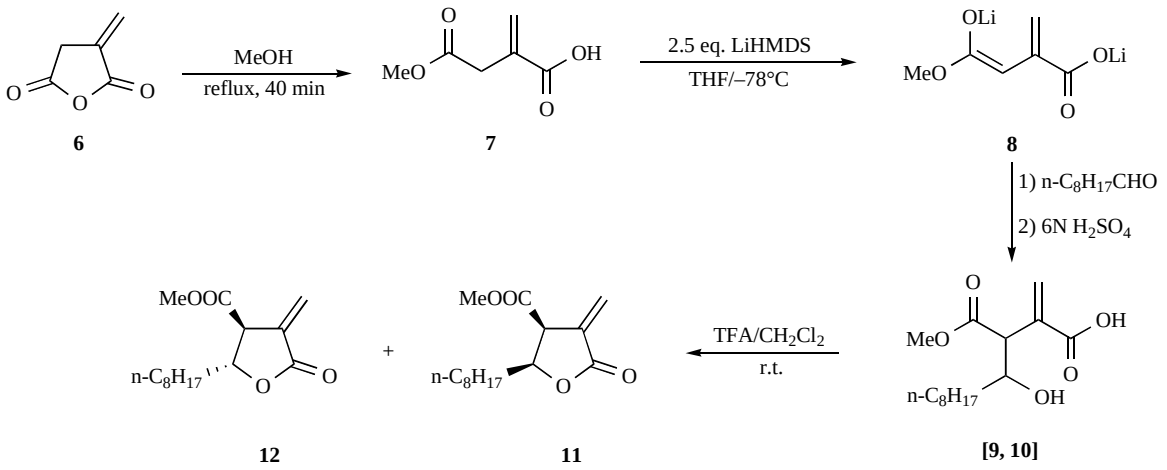


Scheme 1. Synthesis of racemic methyl ester of C75, (±)-(**12**) and its diastereomer (±)-(**11**).

lactonization into the corresponding *cis* and *trans* lactonic esters (**11**) and (**12**) [23] respectively was accomplished with TFA (0.5 eq) in CH_2Cl_2 at room temperature. Diastereomers (**11**) and (**12**) were separated by flash chromatography (23% and 18% total yield respectively).

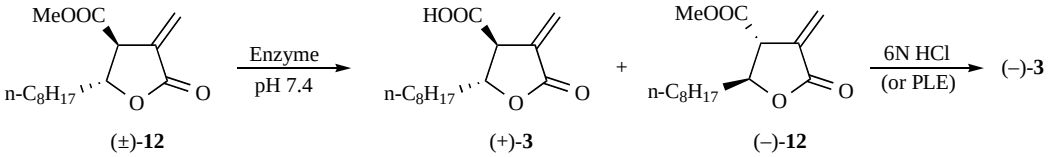
Enzymatic Hydrolyses of the Methyl Ester of C75

Following the general criteria in developing the most suitable procedure for kinetic enzymatic resolution [24] of the *trans* lactone (±)-(**12**) (Scheme **2**), several enzymes were screened. Lipases such as Porcine pancreatic lipase (PPL) and Lipase from *Aspergillus niger* (AP12), as well as a protease such as α-chymotrypsin (α-CT) were inactive. Esterases, such as horse liver acetone powder (HLAP) and purified Pig liver esterase (PLE), hydrolyzed regioselectively the methyl ester group of compound (±)-(**12**) but without any enantioselection. The rate of hydrolysis was higher for PLE than for HLAP.

On the contrary, hydrolysis proved enantioselective when performed with two Acylases I. The results obtained using Acylases I immobilized on Eupergit® C from *Aspergillus* sp. and Amano Acylase from *Aspergillus melleus* are reported in Table **1**. Acylase I from porcine kidney, Grade I, was also checked but without any result.

The reaction was scaled up using both Acylases. We observed that while with Acylase I immobilized on Eupergit® C, the enantiomeric ratio *E* decreased from 60 to 20, that of Amano Acylase increased from 22 to 41. In the former case, the acid (+)-(**3**) was obtained with 86% e.e. stopping the reaction at about 50% conversion [28]. After recrystallization from light petroleum, its e.e. raised to 98% (white solid, m.p. 88–90 °C [lit. [2a] m.p. 76–77 °C for the racemic mixture], $[\alpha]_D^{25}$ = +8.4 (*c* 0.15, MeOH), CD in MeOH: $\Delta\varepsilon_{258}$ +0.26, $\Delta\varepsilon_{225}$ –10.58). The unreacted ester (–)-(**12**) was recovered with 77% e.e.

With Amano Acylase at 56% conversion value, [29] the recovered unreacted ester (–)-(**12**) had >99% e.e. ($[\alpha]_D^{25}$ = –



Scheme 2. Enzymatic resolution of the methyl ester of C75.

Table 1. Enantioselective Hydrolyses of (±)-(12) with Acylases I

Entry	Enzyme	Time	Conv.[a] (%)	(+)-(3) e.e. (%) [b]	(–)-(12) e.e. (%)[c]	*E*[d]
1	Acylases I, immobilized on Eupergit® C[e]	5h	18	96	21	60
2	Amano Acylase[f]	4h	17	90	18	22

[a]Calculated values, Ref. [25].
[b]Determined by chiral HRGC on a Chiraldex™ type G-TA, trifluoroacetyl γ-cyclodextrin column (40 m x 0.25 mm) (carrier gas He, 180 KPa, split 1:100) of its ethyl ester [26] Rt 183.6 min for (2*R*,3*S*)-(+)-enantiomer and R_t 201.7 min for (2*S*,3*R*)-(–)-enantiomer (150°C).
[c]Determined by chiral HRGC on a Chiraldex™ type G-TA, trifluoroacetyl γ-cyclodextrin column (40 m x 0.25 mm) (carrier gas He, 180 KPa, split 1:100) R_t 155.6 min for (2*R*,3*S*)-(+)-enantiomer and R_t 164.5 min for (2*S*,3*R*)-(–)-enantiomer (150 °C).
[d]Ref. [27].
[e]Conditions: compound (±)-(**12**) (0.034 g, 0.12 mmol), 0.1 M phosphate buffer (10 mL) at pH 7.4, 1 mM $CoCl_2$, 0.1 g of Acylase I immobilized on Eupergit® C 102 U/g, r.t.
[f]Conditions: compound (±)-(**12**) (0.050 g, 0.19 mmol), 0.1 M phosphate buffer (10 mL) at pH 7.4, 0.05 g of Amano Acylase 30000 U/g, r.t.

7.1 (*c* 0.42, MeOH), CD in MeOH: $\Delta\varepsilon_{258}$ –0.30, $\Delta\varepsilon_{223}$ +9.3), while the acid (+)-(**3**) was isolated with 78% e.e.

With the purpose of obtaining the enantiomeric lactonic acid (–)-(**3**), the enantiopure lactonic ester (–)-(**12**) having >99% e.e. was subjected to both chemical and enzymatic hydrolyses. Chemical hydrolysis was performed with 6N HCl [18k,30], while Pig liver esterase (PLE) was used for enzymatic hydrolysis. [31] In both cases the enantiomeric excess of the ester was retained in the resulting acid and optically pure (–)-C75 was obtained with >99% e.e. (m.p. 88–90 °C, $([\alpha]_D^{25} = -9.5$ (*c* 0.49, MeOH)).

The (2*R*,3*S*) absolute configuration was assigned to the dextrorotatory enantiomer of C75 by comparison of its CD spectrum ($\Delta\varepsilon_{258}$ +0.26, $\Delta\varepsilon_{225}$ –10.58) with that of (2*R*,3*S*)-(+)-protolichesterinic acid (**5**) ($\Delta\varepsilon_{257}$ +0.32, $\Delta\varepsilon_{220}$ –9.62) [32], whose absolute configuration is known. On the other hand, the positive sign of its specific optical rotation was already indicative that the absolute configuration of (+)-C75 was the same as that of (+)-methylenolactocin (**2**), (+)-nephrosterinic acid (**4**) and (+)-protolichesterinic acid (**5**), namely (2*R*,3*S*) [18e].

CONCLUSION

Two cases are reported in the literature on enzymatic resolution of α-methylene-γ-lactones [33], both bearing the alkoxycarbonyl group at the γ-position. It is known that in the presence of a carboxy group the β-position greatly favors the exo-endo equilibration of the double bond [19], thus preventing any chemical resolution. The enzymatic resolution reported here is of particular interest.

Furthermore, this work represents another interesting example of the ability of acylases to hydrolyze the ester group [34].

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[23] *Cis* lactone **11**: ^{1}H NMR (δ ppm, 400 MHz): 6.40 (1H, d, J=2.2 Hz, C=C*H*$_2$), 5.83 (1H, d, J=2.2 Hz, C=C*H*$_2$), 4.63 (1H, ddd, J_1=4.2, J_2=7.9, J_3=9.0 Hz, H-2), 4.01 (1H, dt, J_1=J_2=2.2, J_3=7.7 Hz, H-3), 3.76 (3H, s, OCH$_3$), 1.60 (2H, m), 1.4-1.2 (12H, m), 0.88 (3H, t, J=6.8, CH$_3$); ^{13}C NMR (δ ppm, 67.80 MHz) 169.3 (s), 168.8 (s), 133.6 (s, C-4), 124.9 (t, C=*C*H$_2$), 79.0 (d, C-2), 52.3 (q, OCH$_3$), 49.1 (d, C-3), 31.7 (t), 31.5 (t), 29.3 (t), 29.2 (t), 29.1 (t), 25.5 (t), 22.6 (t), 14.0 (q). *Trans* lactone **12**: ^{1}H NMR (δ ppm, 400 MHz): 6.40 (1H, d, J=2.8 Hz, C=C*H*$_2$), 5.91 (1H, d, J=2.8 Hz, C=C*H*$_2$), 4.80 (1H, q, J=6.2 Hz, H-2), 3.80 (3H, s, OCH$_3$), 3.59 (1H, dt, J_1=J_2=2.8, J_3=6.2 Hz, H-3), 1.72 (2H, m), 1.5-1.2 (12H, m), 0.88 (3H, t, J=6.5, CH$_3$); ^{13}C NMR (δ ppm, 67.80 MHz) 169.7 (s), 168.3 (s), 133.0 (s, C-4), 125.1 (t, C=*C*H$_2$), 79.0 (d, C-2), 52.9 (q, OCH$_3$), 49.7 (d, C-3), 35.7 (t), 31.7 (t), 29.3 (t), 29.1 (2t), 24.7 (t), 22.6 (t), 14.0 (q).

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[26] 0.005 g of (+)-**3** was dissolved in ethanol, 20 µl of TMSCl were added and the solution was stirred overnight. After evaporation of the solvent, 1 mL of CH_2Cl_2 was added and the solution was analysed on chiral HRGC (γ-CDX).

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[28] Compound (±)-**12** (0.450 g, 1.7 mmol) was added to a suspension of Acylase I immobilized on Eupergit® C (1.5 g, 153 U) in a 1 mM solution of $CoCl_2$ (150 mL of phosphate buffer at pH 7.4). After stirring for 24 h, maintaining the pH value constant by addition of 1N NaOH, the mixture was extracted with ether at pH 7.4. The solvent extracted both the acid and the unreacted ester, so they needed to be separated. The organic phase was then treated with a 5% $NaHCO_3$ solution. The organic phase containing the ester was dried on anhydrous Na_2SO_4 and, after elimination of the solvent under vacuum, compound (-)-**12** was obtained with 77% e.e. (0.190 g, 42% yield). The basic aqueous phase was acidified to pH 2 with HCl, extracted with ether and dried on anhydrous Na_2SO_4. Evaporation of the solvent afforded the acid (+)-**3** (0.127 g, 30% yield, 86% e.e.) as a white solid. The solid was refluxed in petroleum ether, the acid (+)-**3**, recovered by decantation of the hot solvent, had 98% e.e, m.p. 88-90°C, $[\alpha]_D^{25}$ = +8.4 (c 0.15, MeOH), CD in MeOH $\Delta\varepsilon_{258}$ +0.26, $\Delta\varepsilon_{225}$ -10.58.

[29] Compound (±)-**12** (0.239 g, 0.9 mmol) was added to a suspension of Amano Acylase (0.239 g, 7170 U) in phosphate buffer at pH 7.4 (40 ml), the mixture was stirred for 48 h, while maintaining the pH value constant by addition of 1N NaOH, and extracted with ether. The acidic compound (+)-**3** with 78% e.e. (28% yield) was separated and the recovered ester (-)-**12** was found to have >99% e.e., 34% yield, $[\alpha]_D^{25}$ = -7.1 (*c* 0.42, MeOH), CD in MeOH: $\Delta\varepsilon_{258}$ -0.30, $\Delta\varepsilon_{223}$ +9.3.

[30] Compound (-)-**12** (0.085 g, 0.32 mmol) with 99% e.e. was refluxed in 2-butanone with 12 drops of 6N HCl. After 4 hours the solvent was evaporated and the solid mass was extracted with 5% $NaHCO_3$ solution to separate the acid from the unreacted ester. The aqueous layer, acidified to pH 2, was then extracted with diethyl ether, the organic layer was dried over anhydrous Na_2SO_4. Evaporation of the solvent under vacuum furnished the acidic lactone (-)-**3** (0.068 g, 82% yield), m.p. 88-90ºC, $[\alpha]_D^{25}$ = -9.5 (c 0.49, MeOH), >99% e.e.

[31] Compound (-)-**12** (0.083 g, 0.31 mmol) with 99% e.e. in acetone (3 mL) and phosphate buffer, pH 7.4 (27 mL) was stirred with 6 mg PLE (4839 U/mmol) at room temperature for 23 hr maintaining the pH value constant by adding 1N NaOH solution. After the reaction was complete the reaction mixture was extracted with ether at pH 7.4. The organic layer was dried over anhydrous Na_2SO_4, the solvent was removed and (-)-**3** was recovered (>99% e.e., 0.056 g, 71% yield).

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