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Preparation and high-performance liquid chromatographic analysis of *syn* and *anti* isomers of steroidal 3-(O-carboxymethyl) oximes

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ABSTRACT

Syn and *anti* isomeric mixtures of methyl esters of 3-(O-carboxymethyl) oximes of progesterone, 17-α-OH-progesterone, testosterone, and cortisol were synthesized and separated on a preparative scale. The pure esters were then hydrolyzed to afford the desired *syn* or *anti* 3-(O-carboxymethyl) oximes. Analysis by HPLC was developed to determine isomeric and chemical purity of the acids and esters.

INTRODUCTION

The O-carboxymethyl oxime (CMO) functionality has been used for various conjugates for over 35 years [1,2]. Steroidal CMOs are widely used in the preparation of conjugates for immunoassays which quantify biologically important steroids. CMOs can exist as two different geometric isomers, *syn* or *anti* (Fig. 1). Most of the biologically important steroidal CMOs are commercially available from different vendors, however, the isomeric purity is never specified. Fractional crystallization has been employed to obtain a single or enriched isomer with some success, though, significant amounts of material were sacrificed [3–6]. Mappus *et al.* [7] reported on the separation of *syn* and *anti* isomers by TLC (silica gel) of several steroidal 3-CMOs using the corresponding methyl esters. Mitsuma *et al.* [8] separated the two isomers of progesterone 3-CMO N-hydroxysuccinimide esters by column chromatography (silica gel) and the resulting "active" esters were useful for direct conjugation to proteins. Both reports, however, failed to include the chemical yield and isomeric purity, and they expressed concern about racemization during ester hydrolysis and work-up to the free acid. Also, a direct chromatographic method to estimate isomeric purity of the final CMO product was not reported.

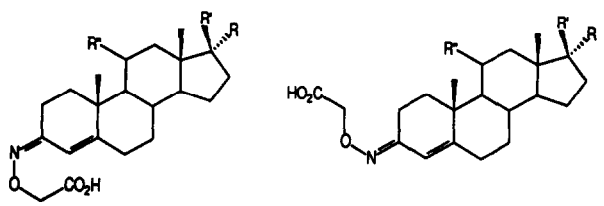

Fig. 1. *Syn* and *anti* isomers of 3-(O-carboxymethyl) oximes of progesterone (1), 17-α-OH-progesterone (2), testosterone (3), and cortisol (4). R, R′, R″: (1) OH, C(O)Me, H; (2) H, C(O)Me, H; (3) H, OH, H; (4) OH, C(O)CH_2OH, OH.

We needed chemically and isomerically pure (both isomers) steroidal CMOs to meet the expectations of good manufacturing practices (GMP, ISO 9000) [9,10]. Additionally, a simple

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method to directly assess the chemical and isomeric quality was required.

In this paper we report the preparation of *syn* and *anti* isomers of 3-(O-carboxymethyl) oximes of progesterone (**1**), 17-α-OH-progesterone (**2**), testosterone (**3**), and cortisol (**4**). We also describe an HPLC method to resolve and quantify the isomers.

EXPERIMENTAL

General

Solvents were HPLC grade [Fisher (Pittsburgh, PA, USA) or EM Science (Gibbstown, NY, USA)] and used without further purification. Progesterone, cortisol, 17-α-hydroxyprogesterone, testosterone 3-CMO, and N-methyl-N′-nitro-N-nitrosoguanidine were purchased from Sigma (St. Louis, MO, USA). Triethylamine, methyl chloroformate, and 4-dimethylaminopyridine were purchased from Aldrich (Milwaukee, WI, USA). HPLC analysis was performed using a custom L.C. (Custom L.C., Houston, TX, USA) C_{18}, 3 μm, 80 Å, 150 × 6.2 mm column at room temperature, eluting at flow-rate of 1 ml/min. Retention times and mobile phase are recorded in Table I.

General synthesis

O-Carboxymethyl oximes. 3-(O-Carboxymethyl) oximes of 17-α-OH-progesterone, progesterone, and cortisol (testosterone was purchased as the 3-CMO) were prepared using a previously described method [6].

Methyl esters. The crude CMO was dissolved in dry methylene chloride (100 m*M*) and cooled with an ice bath. To the clear solution was added 1.5 equivalents of triethyl amine and methyl chloroformate (added dropwise), and 0.1 equivalents of 4-dimethylaminopyridine. Upon depletion of the acid [TLC, ethyl acetate–hexane (1:3); (3:2) for cortisol] the organic layer was washed with 1 *M* sodium bicarbonate and water. The heterogeneous mixture was allowed to stand overnight and then the organic layer was collected, dried (magnesium sulfate) and concentrated by reduced pressure. Separation of the *syn* and *anti* esters was accomplished by gravity chromatography [50 × 5 cm glass column, 300 g silica gel, 230–400 mesh (40–63 μm particle size), 60 Å]. Elution was with ethyl acetate–hexane (1:3). Pure fractions of each isomer were pooled and concentrated under vacuum affording white solids. [All synthetic products gave correct analytical data (^{1}H and ^{13}C NMR, MS and HPLC)]

An alternative method was used for cortisol. The CMO was dissolved in methanol (100 m*M*) and cooled to −5°C with an ice/salt bath. Diazomethane [11], prepared just prior to use, was added in four portions (10-fold excess) as an ether solution. The yellow solution was allowed to warm to room temperature and then stirred overnight to afford a clear solution. The solvents were removed under reduced pressure to afford

TABLE I

MOBILE PHASE USED FOR HPLC ANALYSIS AND RETENTION TIMES FOR *syn* AND *anti* ISOMERS OF 3-(O-CARBOXYMETHYL) OXIMES **1–4**

CMO[a]	Retention time (min)				Mobile phase[b] water–methanol
	syn Acid	*anti* Acid	*syn* Ester	*anti* Ester	
1	15.7	17.4	32.3	36.5	25:75
2	16.4	18.1	27.5	31.6	3:7
3	15.5	16.9	28.5	32.8	3:7
4	29.8	28.5	35.6	34.9	6:4 to 3:7 in 35 min[c]

[a] See Fig. 1 for definition.
[b] Phase contains 0.1% trifluoroacetic acid.
[c] Linear gradient.

TABLE II

ISOLATED YIELD AND ISOMERIC PURITY OF *syn* AND *anti* STEROIDAL 3-(O-CARBOXYMETHYL) OXIMES **1–4**

CMO	Isolated yield (%) and isomeric purity[a]			
	syn Ester	*anti* Ester	*syn* Acid[b]	*anti* Acid[b]
1	25	46	95 (98)	92 (99)
2	26	45	94 (97)	94 (99)
3	16	47	91 (94)	88 (99)
4	7	43	60 (74)[c]	68 (98)

[a] Isomeric purity was >99% for each ester and is listed in parentheses for the acids.
[b] Yield based from pure ester.
[c] The *syn* cortisol CMO was not a stable isomer and was prone to rapid isomerization to the *anti* during work-up.

the desired 3-CMO methyl esters. Separation of the *syn* and *anti* esters was carried out as described above except elution was with ethyl acetate–hexane (3:2).

Hydrolysis

The separated esters were treated with methanolic 2 *M* sodium hydroxide (0.1 ml/mg of ester). Typical hydrolysis took 2 hours (followed by TLC) at room temperature. Upon completion the reaction mixture was made acidic (pH 4.0) with 2 *M* HCl. The CMO was extracted out with ethyl acetate and the organic layer was washed with water until neutral pH was achieved (very important to remove all residual HCl). After drying with $MgSO_4$ the solvent was removed under reduced pressure to afford the desired 3-CMO. Table II lists the isolated yield and isomeric purity.

RESULTS AND DISCUSSION

The steroidal 3-CMOs prepared herein are available from different vendors, unfortunately their isomeric purity is not specified. Based on HPLC analysis, we found the chemical purity and the *syn*:*anti* ratios to fluctuate between manufacturers and from lot to lot, even from the same company. Attempts in our lab to obtain significant amounts (>100 mg) of the pure isomers by recrystallization and/or reversed-phase HPLC separation of purchased material became costly and inefficient. Recently we reported an enzyme-mediated hydrolysis of the 3-CMO methyl esters of **1–4** using lipase from *Candida cylindracea* which resulted in 80–98% isomeric purity on an analytical (<1 mg) scale [12].

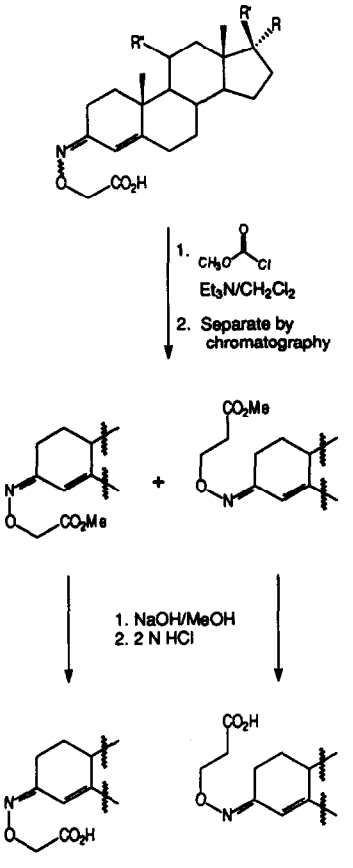

Fig. 2. General route for the preparation of *syn* and *anti* isomers of steroidal 3-(O-carboxymethyl) oximes **1–4**.

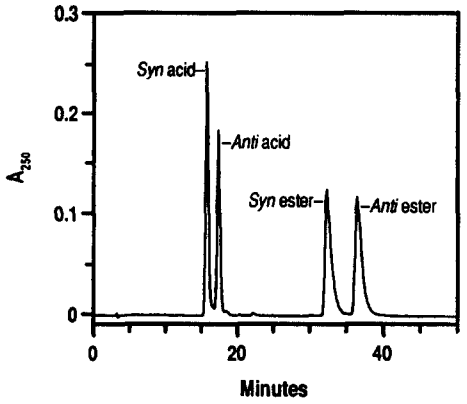

Fig. 3. HPLC of **1**, acids and esters, which is representative for all steroidal 3-CMOs **1–4**.

We were interested in obtaining each CMO isomer on a gram scale. We developed a cost effective method which involves derivatization of the parent steroid and then separation of the CMO methyl esters (Fig. 2). Preparation of the steroidal 3-CMOs was taken from literature protocols [3]. Without purification the CMOs were directly converted to methyl esters using either methyl chloroformate or diazomethane and the resulting esters were then purified by gravity chromatography on silica gel. Base hydrolysis was employed to reconstitute the steroidal CMO. Great care was taken to remove all traces of mineral acid used during the work-up to minimize acid-catalyzed racemization.

The isomers of the acids and esters could be completely resolved (Fig. 3) on a reversed-phase HPLC column using water–methanol–trifluoroacetic acid elution (Table I) affording a convenient method to determine purity.

As has been reported [6,7], the CMOs are susceptible to racemization under mild conditions (methanol). A slight loss of isomeric purity (Table II) was unavoidable and is attributed to the acid-catalyzed isomerization during work-up. It was possible to completely isomerize either isomer in methanol in approximately ten days. However, in a crystalline state, the CMOs retain stereo integrity after 6 months when stored at room temperature.

In summary, using this strategy of chemical derivatization followed by column chromatography and final hydrolysis it was possible to obtained gram quantities of isomerically pure ($\geq 94\%$) and chemically pure ($\geq 99.5\%$) steroidal 3-CMOs of **1–4** in high yield. The utility of the outlined method permits the synthesis of high quality CMOs and the ability to directly determine the chemical and isomeric purity of these important conjugate precursors.

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