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Novel stereoselective synthesis and chromatographic evaluation of *E*-guggulsterone

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ABSTRACT

A new stereoselective synthesis of *E*-guggulsterone is described starting from androsten-3,17-dione. Protection of the ring A enonic system, followed by regioselective Wittig reaction and C-16 oxidation, affords *E*-guggulsterone in good yields and high stereoselectivity, making this approach easily accessible and scalable. Moreover, an original normal-phase HPLC method enabling the fast quantitation of the guggulsterone isomeric purity, combined with the suitability for sampling procedures, is detailed. The relying upon the cellulose-based Chiralpak IB column and the chloroform as the "non-standard" component of the eluent mixture, allows to get profitably high chromatographic performances.

1. Introduction

The herbal extract guggulipid from the tree *Commiphora mukul* has been used in Indian Ayurvedic medicine for more than 2000 years to treat a variety of ailments including diabetes, atherosclerosis, as well as osteoarthritis and inflammation [1,2]. The gum resin, which is available on the market since 1988 as a potent hypolipidaemic agent, is a complex mixture of diverse classes of compounds, such as lignans, lipids, diterpenoids and steroids [3]. Among these, two steroidal isomers known as *E*- and *Z*-guggulsterone (*E*- and *Z*-4,17(20)-pregnadiene-3,16-dione, **1** and **2**) (Fig. 1) are considered as the key active ingredients responsible for guggul's therapeutic effects. Guggulsterones have been reported to lower lipids and cholesterol levels [4–6], to be useful in the treatment of various cardiovascular diseases [7], and to be endowed with anti-neoplastic properties [8].

A number of studies have shown that the biological activity of guggulsterones is at least partly due to their action as ligands of the farnesoid X receptor (FXR) [9–11]. Both isomers **1** and **2** were found to selectively modulate FXR gene expression and, in particular, to positively regulate the expression of the cytochrome Cyp7A1, thus inducing the cholesterol catabolism in bile acids and lowering cholesterol levels. Following experimental evidences, however, indicated that additional pathways are involved in the pharmacological action of guggulsterones. In fact, they may exert their biological effects by the modulation of transcriptor factors as nuclear factor kappa B (NFκB), STAT-3 and C/EBP alpha [12–14], as well as of endocrine steroid and metabolic lipid receptors [15,16].

On the basis of these considerations, there is a strong demand for guggulsterones to better define their biological mechanisms and clinical significance. Being not easily available by extraction procedures (yield: 1.1%) because of their low content in the gum resin, the availability of guggulsterones **1** and **2** comes mainly from synthetic preparations. The first synthesis was reported by Benn and Dodson in 1964 [17], even before their isolation from guggulipids [18]. The method involves the use of 16-dehydropregnenolone acetate (**3**, 16-DIPA) (Fig. 1) as starting material, which is not commercially available and needs to be synthesized besides the low overall yields. In the course of recent years, a number of patents claims improved protocols for the preparation of isomeric mixture of guggulsterones [19,20], while very recently, a regioselective synthesis of *E*-guggulsterone (**1**) has been described from 16,17-epoxy-pregnenolone (**4**) (Fig. 1) via hydrazine reduction and Oppenhauer oxidation [21].

As a part of our ongoing research program in the field of FXR modulators [22] and in the synthesis of natural bioactive steroids [23], herein we report a new, efficient and gram scale regioselective synthesis of *E*-guggulsterone as well as the description of a valuable HPLC protocol for the chromatographic evaluation of both isomers **1** and **2**. At this regard, attempts to obtain the *Z*-isomer **2** from **1** are also described.

Abbreviations: GS, guggulsterone; FXR, farnesoid X receptor.

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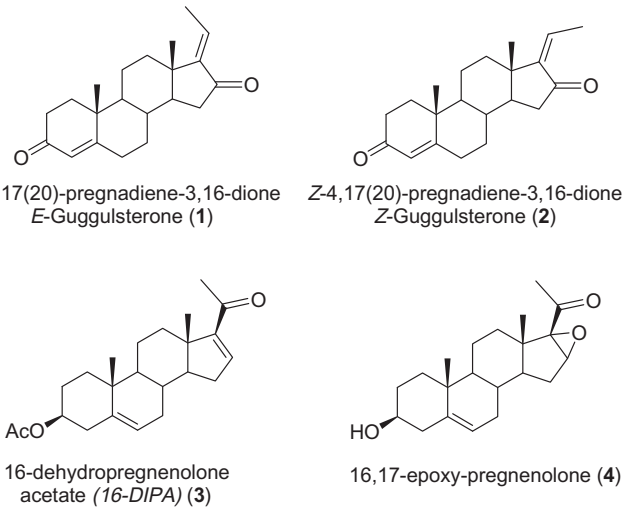


Fig. 1. Structure of *E*- and *Z*-guggulsterone, and related synthetic precursors.

2. Experimental

2.1. Materials

All reagents were commercially available unless otherwise noted. The final products were purified by chromatography on silica-gel (70–230 mesh). TLC was performed on aluminum backed silica plates (silica gel 60 F_{254}). Spots on TLC were visualized by using UV and by staining and warming with phosphomolybdate reagent (5% solution in EtOH). All the reactions were performed using distilled solvents. ^{1}H NMR spectra were recorded at 200 and 400 MHz, ^{13}C NMR spectra were recorded at 100.6 MHz, using the solvents indicated below. Chemical shifts are reported in parts per million (ppm). The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; q, quartet; psd, pseudo singlet. Melting points were determined with an electrothermal apparatus and are uncorrected. Optical rotations were measured with a Jasco Dip-1000 polarimeter in $CHCl_3$.

2.2. Synthesis

2.2.1. 3-Ethoxyandrosta-3,5-dien-17-one (**6**)

Triethyl orthoformate (2.60 mL, 15.63 mmol) and *p*-toluensulfonic acid (0.027 g, 0.14 mmol) were added to a stirred solution of androsten-3,17-dione (**5**) (2.00 g, 6.98 mmol) in freshly distilled THF (20 mL) and absolute EtOH (0.64 mL) under N_2 atmosphere. The resulting mixture was heated at 45 °C for 2 h. The reaction was quenched with 10% $NaHCO_3$ (5 mL) and extracted with Et_2O (3 × 15 mL). The collected organic layers were washed with brine (10 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, to give **6** in almost quantitative yield (2.19 g, 6.97 mmol). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.90 (s, 3H, 18-CH_3), 0.99 (s, 3H, 19-CH_3), 1.24–1.31 (m, 3H, OCH_2CH_3), 3.73–3.81 (m, 2H, OCH_2CH_3), 5.11 (s, 1H, 4-C*H*), 5.21 (s, 1H, 6-C*H*). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ: 13.6, 14.6, 18.9, 20.4, 21.8, 25.4, 30.7, 31.4, 31.5, 33.7, 35.2, 35.8, 47.6, 48.4, 51.91, 62.1, 98.8, 117.0, 141.21, 154.6, 221.0.

2.2.2. (17Z)-pregna-4,17-dien-3-one (**8**)

Potassium *t*-butoxide (3.02 g, 26.90 mmol) was added to a stirred solution of ethyl-triphenylphosphonium bromide (6.56 g, 17.62 mmol) in distilled THF (50 mL) and under N_2 atmosphere. The resulting suspension was reacted at 60 °C for 90 min. A solution of **6** (1.09 g, 3.49 mmol) in THF (25 mL) was then added dropwise and reacted at 60 °C for 18 h. The reaction mixture was cooled at room temperature, diluted with H_2O (30 mL) and extracted with Et_2O (3 × 25 mL). The collected organic layers were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude was dissolved in THF (50 mL) and treated with HCl 37% (1 mL) at room temperature for 2 h. The mixture was diluted with EtOAc (30 mL), washed with 10% $NaHCO_3$ (2 × 20 mL), brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography with petroleum ether/EtOAc to give **8** in 95% yield (0.98 g, 16.73 mmol). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 0.91 (s, 3H, 18-CH_3), 1.18 (s, 3H, 19-CH_3), 1.63 (d, 3H, J = 7.1 Hz, 21-CH_3), 5.07–5.18 (m, 1H, 20-C*H*), 5.72 (s, 1H, 4-C*H*). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ: 13.0, 16.7, 17.3, 20.9, 24.1, 31.2, 31.8, 32.8, 33.9, 35.1, 35.6, 36.8, 38.6, 44.0, 53.7, 55.5, 113.6, 123.7, 149.5, 171.4, 199.5.

2.2.3. (17E)-16α-hydroxy-pregna-4,17-dien-3-one (**9**)

t-Butylhydroperoxide (TBHP) (0.16 mL, 0.88 mmol) was added to a stirred suspension of SeO_2 (0.041 g, 0.37 mmol) in distilled CH_2Cl_2 (3 mL) under N_2 atmosphere at 0 °C. The resulting mixture was reacted for 30 min. A solution of **8** (0.022 g, 0.74 mmol) in CH_2Cl_2 (3 mL) was then added dropwise, warmed to room temperature and reacted at this temperature overnight. The reaction was quenched with $NaHCO_3$ 10% (5 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The collected organic layers were washed with brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography with petroleum ether/EtOAc to give 0.208 g (0.66 mmol, 90%) of **9**. mp: 132–134 °C. $C_{21}H_{30}O_2$: Calcd. C, 80.21; H, 9.62. Found. C, 80.30; H, 9.30. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.89 (s, 3H, 18-CH_3), 1.16 (s, 3H, 19-CH_3), 1.71 (d, 3H, J = 7.2 Hz, 21-CH_3), 4.42 (br d, 1H, J = 4.0 Hz, 16-C*H*), 5.57 (q, 1H, J = 7.2 Hz, 20-C*H*), 5.71 (s, 1H, 4-C*H*). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ: 13.2, 17.2, 17.3, 21.0, 31.7, 32.7, 33.9, 34.4, 34.9, 35.5, 36.9, 38.5, 44.1, 51.8, 53.7, 74.1, 119.8, 123.8, 154.6, 171.1, 199.5.

2.2.4. E-guggulsterone (**1**)

Oxalyl chloride (0.064 mL, 0.74 mmol) was added to a stirred solution of DMSO (0.11 mL, 1.49 mmol) in distilled CH_2Cl_2 (3 mL) under N_2 atmosphere at −50 °C. After 10 min a solution of **9** (195 mg, 0.62 mmol) in CH_2Cl_2 (3 mL) was added dropwise and the mixture was reacted at −50 °C for 40 min. Then Et_3N (0.43 mL, 3.10 mmol) was added, and the resulting reaction was stirred at −50 °C for 40 min, at −20 °C for additional 2 h, and finally warmed at room temperature. The mixture was diluted with CH_2Cl_2 (10 mL) and treated with HCl 3 N (10 mL) for 10 min. The organic phase was separated, washed with H_2O (5 mL), $NaHCO_3$ 10% (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography with petroleum ether/EtOAc to give the desired *E*-guggulsterone (**1**) (0.165 g, 0.52 mmol, 85%). R_f (petroleum ether/EtOAc-7:3, v/v) 0.18. mp: 169–171 °C. $C_{21}H_{28}O_2$: Calcd. C, 80.73; H, 9.03. Found. C, 79.89; H, 8.88. $[\alpha]_D^{20}$ −27.4° (c = 0.016, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.08 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 1.86 (d, 3H, J = 7.3 Hz, 21-CH_3), 5.75 (s, 1H, 4-C*H*), 6.52 (q, 1H, J = 7.5 Hz, 20-C*H*). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ: 13.1, 17.3, 17.5, 20.6, 31.8, 32.5, 33.8, 34.2, 35.4, 35.9, 37.7, 38.6, 43.0, 49.5, 53.3, 124.1, 129.5, 147.4, 170.1, 199.2, 205.6.

2.2.5. General procedure for the synthesis of Z-guggulsterone (**2**)

E-guggulsterone (**1**) (0.16 mmol) was dissolved in the appropriate solvent (10 mL) and treated with the catalyst (0.3 equivalents). The mixture was stirred at room temperature for 7 h. The reaction mixture was filtered (when needed), washed with 10% $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated under

reduced pressure. The residue was purified by silica gel flash chromatography using a solution of petroleum ether-EtOAc (from 9:1 to 7:3, v/v) as eluent to give the pure *Z*-guggulsterone (**2**). mp: 191–192 °C. R_f (petroleum ether/EtOAc-7:3, v/v) 0.25. $C_{21}H_{28}O_2$: Calcd. C, 80.73; H, 9.03. Found. C, 79.72; H, 8.26. $[\alpha]_D^{20}$ −50.8° (c = 0.016, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.97 (s, 3H, 18-C*H*$_3$), 1.23 (s, 3H, 19-C*H*$_3$), 2.07 (d, 3H, *J* = 7.2 Hz, 21-C*H*$_3$), 5.71–5.76 (m, 2H, 4-C*H* + 20-C*H*). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ: 14.0, 17.3, 19.5, 20.6, 31.7, 32.5, 33.9, 34.5, 35.4, 35.5, 38.7, 39.2, 43.0, 48.9, 53.5, 124.1, 130.5, 147.8, 170.3, 199.3, 207.8.

2.3. HPLC analysis

The HPLC separation of guggulsterone isomers has been carried out with the column Chiralpak IB (250 × 4.6 mm I.D., containing cellulose tris(3,5-dimethylphenylcarbamate) immobilized onto a 5 μm silica gel) which was purchased from Chiral Technologies (West Chester, PA, USA). Before the use, the selected column has been conditioned with the selected mobile phase at a 1.0 mL/min flow rate for at least 40 min. All the analyses have been run at 1.0 mL/min flow rate, with a 20 °C column temperature, and monitored at 220 nm. The analytical HPLC measurements have been made on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 20 μL stainless steel loop. Column temperature has been controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R) thermostat. The 1,3,5-triisopropylbenzene has been used as the unretained marker to evaluate on a model isomeric mixture the chromatographic performance with the two eluent systems. The corresponding retention time (t_0) has been found to be 3.26 min. Before the use, all the employed mobile phases have been degassed through sonication. Analytes to be injected have been solubilized in the selected mobile phase.

2.3.1. Selected chromatographic parameters

All the following chromatographic parameters have been calculated according to the German Pharmacopeia (DAB). The retention factor (k) values have been computed by taking the retention time (t_R) at the peak maximum. Separation factor (α) and resolution factor (R_S) values have been respectively computed from the following Eqs. (1) and (2):

$$\alpha = \frac{k_2}{k_1} \tag{1}$$

$$R_s = 1.18\frac{t_R - t_{Rp}}{W_{0.5} + Wp_{0.5}} \tag{2}$$

where k_1 is the retention factor of the first eluted isomer, k_2 is the retention factor of the second eluted isomer, $W_{0.5}$ is the width of the peak at the position of 50% peak height, $Wp_{0.5}$ is the width of the peak at the position of previous 50% peak height and t_{Rp} is the retention time of the first eluted peak within each isomer couple.

3. Results and discussion

3.1. Synthesis

The synthetic strategy employed to access *E*-guggulsterone (**1**) was based on the use of androsten-3,17-dione (**5**) as starting material (Scheme 1). Thus, in order to prevent side-reactions on the enone system at ring A, **5** was initially treated with triethyl orthoformate in the presence of catalytic amounts of *p*-toluen sulfonic acid (*p*-TSA) in a mixture of THF/EtOH-30:1 (v/v) to furnish the corresponding enol ether **6** in nearly quantitative yield. The C-17 side chain was generated by Wittig reaction, using the commercially available phosphoran ethyltriphenylbromide and potassium *t*-butoxide (*t*-BuOK) as a base in THF. The reaction, conducted at reflux for 18 h, was followed by acidic hydrolysis to give *Z*-4,17(20)-pregnadiene-3-one (**8**) in 95% yield as a single isomer. The C-16 selective allylic oxidation was reached using selenium dioxide (SeO_2) and *t*-BuO_2H in CH_2Cl_2 at 0 °C affording **9** in 90% yield [24,25]. The correct stereochemistry of (17*E*)-16α-hydroxypregna-4,17-dien-3-one (**9**) was assigned based on both NMR COSY and NOE analysis (Fig. 2) and literature comparison [19,25]. Finally, Swern oxidation and silica gel purification gave 4,17(20)-*cis*-pregnadiene-3,16-dione (**1**) (*E*-guggulsterone, as assigned by Patil et al.) [18] in very good yield (overall yield: 73%).

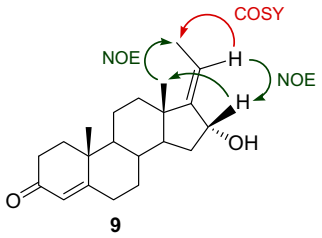
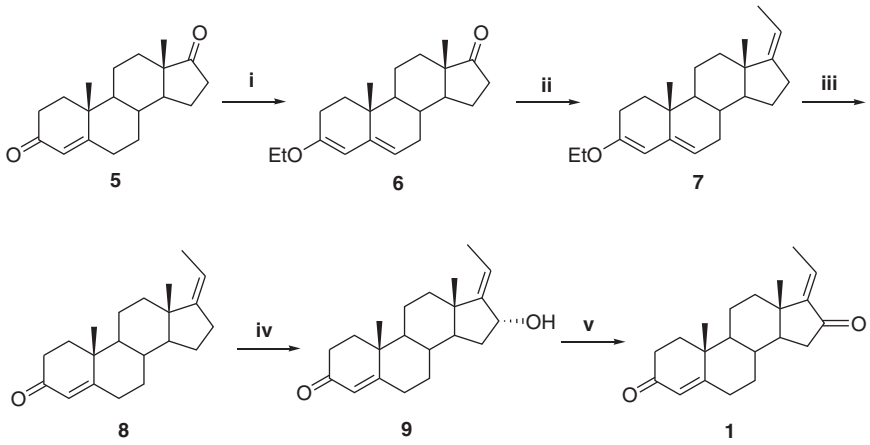
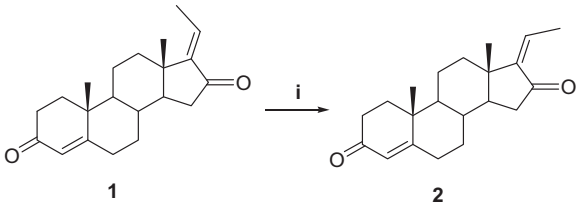


Fig. 2. Diagnostic ^{1}H NMR COSY and NOE for **9**.



Scheme 1. Stereoselective synthesis of *E*-guggulsterone (**1**) from 4-androsten-3,17-dione (5). (i) $CH(OEt)_3$, *p*-TSA, THF/EtOH, quantitative; (ii) $EtPPh_3Br$, *t*-BuOK, THF, reflux; (iii) HCl, THF, 95% from 6; (iv) SeO_2, *t*-BuO_2H, 90%; (v) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, 85%.

Table 1
Isomerization of *E*-guggulsterone (**1**) into *Z*-guggulsterone (**2**).[a]



1 2

Entry	Catalyst	Solvent	Conditions	*Z*:*E* ratio[b]
1	Amberlist-15	Benzene	r.t.	55:45
2	Amberlist-15	Benzene	Sonification	61:39
3	Amberlist-15	Toluene	Sonification	56:44
4	Amberlist-15	CH_2Cl_2	Sonification	48:52
5	$AlCl_3$	CH_2Cl_2	Sonification	48:52
6	*p*-TSA	EtOAc	Reflux	54:46

[a] All the reaction were carried out on a 0.16 mmol scale.
[b] Yield and Z/E ratios were calculated by HPLC analysis.

Next, we have explored the possibility to obtain *Z*-guggulsterone (**2**) by isomerization reaction of *E*-guggulsterone (**1**) using different reaction conditions (Table 1). The best result was obtained with Amberlist 15 ion-exchange resin in benzene under sonication (*E*:*Z* = 39:61). Under these conditions, **2** was isolated in 58% after silica gel purification.

3.2. HPLC estimation of the E- and Z-guggulsterone isomeric purity

Only a very limited number of papers have described the successful HPLC resolution of the *E*- and *Z*-guggulsterone (**1**, **2**). All of these rely upon reversed-phase (RP)-based methods, applied either isocratically [26] or via gradient elution mode [27]. The full validation of the established RP methods has allowed the correct estimation of the guggulsterone isomeric content in both biological fluids and natural products. However, although the recognized usefulness of all these RP-based methods, pre-handling procedures of the material to be analyzed (*viz.* extraction, concentration to dryness, etc.) have been often strictly required before injection into the HPLC column. All these procedures are actually unsuited to a rapid in-line HPLC-based control of the progress of specific synthetic routes or purification steps. Indeed, the direct analysis of the deriving aliquots is frequently complicated by the incompatibility between the water-based eluent phases and the more common synthesis media.

The above limitations have encouraged us to develop a normal-phase (NP) HPLC method enabling the fast quantitation of the isomeric purity of guggulsterones, combined with the suitability for sampling procedures.

Due to the well established broad spectrum of application of the polysaccharide-based stationary phases [28], we have selected a column of this kind for our purpose. In consideration of its universal solvent compatibility, the Chiralpak IB column [28b] has been considered as the elective choice. The possibility to run the analyses in all the chromatographic regimes (even in the presence of "non-standard" eluent components), stems from the covalent grafting of the cellulose-based polymeric units onto an opportunely modified silica gel support [28b]. From a structural standpoint, the three hydroxy groups of each D-glucose unit carry a 3,5-dimethylphenylcarbamate derivatization, which confers a peculiar winding to the polymeric chain. Helical grooves lined with polar carbamate groups exist along the main chain. The carbamate groups, expressing the H-bonding contacts on the polymer (NH and C=O groups), are located in the interior of these cavities, which are delimited by the hydrophobic aromatic rings. As far as the molecular recognition mechanism is concerned, it is commonly assumed to be ascribable to hydrogen bonding and dipole–dipole interactions with the polar carbamate motifs embedded in the chiral cavities, supported by π-stacking and steric interactions contributed by the flanking aromatic groups [28].

It is our belief the polymeric NH groups being potentially involved in H-bonding contacts with the carbonyl moiety at the 16 position on the steroid body, which is in close proximity of the stereo-differentiating side-chain of guggulsterone. This feature has been inferred to be profitably exploited to produce the polysaccharide-based separation of the two guggulsterone isomers.

A series of unsatisfactory analyses preliminarily carried out on a test mixture with conventional NP eluents, has suggested us to attempt the peak resolution by relying upon the presence of non-standard solvents in the mobile phase. Very interestingly, a mobile phase consisting of *n*-hexane/chloroform/IPA-90/8/2 (v,v,v) (system I), has allowed a pronounced resolution of the isomeric peaks (Fig. 3, Table 2). By applying the optimized method, an isomeric purity equal to 98.1% and 98.8% has been established for the *E*- and the *Z*-guggulsterone isomer, respectively (Fig. 3). Such values have been later confirmed by running with a second HPLC method (system II) in which the acetone has been used as the non-standard component into the eluent [*n*-hexane/acetone-85/15 (v,v)] (Table 2). Indeed, a comparable isomeric purity (97.5% and 98.2% for the *E*- and *Z*-guggulsterone isomer, respectively) has turned out.

As evident from a comparison of the results achieved with standard and non-standard eluent components, the mobile phase cannot be merely regarded as a passive transporter of the analytes along the column, but it is rather an essential component inherently involved in the stereoselective between stationary phase-analyte association mechanism. Indeed, the eluent composition influences the degree of complementarities among the interacting

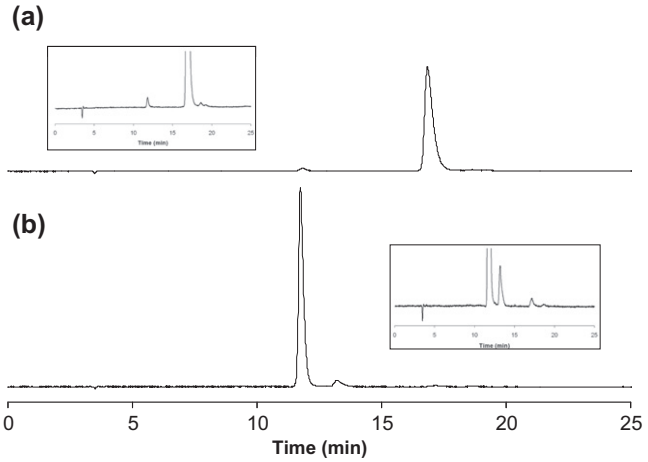


Fig. 3. Chromatographic trace of the (a) *E*- and (b) *Z*-guggulsterone isomer, obtained by running with the *n*-hexane/chloroform/IPA-90/8/2 (v,v,v) containing eluent.

Table 2
Selected chromatographic parameters for separation of *Z*- and *E*-guggulsterone isomers with the two adopted systems (I and II).[a]

System	Isomer	Retention factor (k)	Separation factor (α)	Resolution factor (R_S)
I	*Z*-	2.62	1.61	10.79
	E-	4.23		
II	*Z*-	1.53	1.48	7.98
	E-	2.27		

[a] The chromatographic performance has been evaluated by injecting the 1,3,5-triisopropylbenzene as the unretained marker.

sites on the stationary phase and analyte structure. In fact, the nature of the mobile phase defines the degree of solvation of the immobilized stationary phase and the transient analytes, and thus the "energetic barrier" that must be overcome in the course of the diastereomeric adsorbate formation.

4. Conclusions

A concise and efficient stereoselective synthesis of *E*-guggulsterone (**1**) has been reported. The process provides a straightforward and low cost method for gram scale preparation of pure *E*-guggulsterone (**1**). Advantages of this procedure include the use of low cost and commercially available raw materials, and a good overall yields.

In addition, a relevant isomeric purity (equal to 98.1% for the *E*-guggulsterone isomer) has been established via normal-phase HPLC analysis. Besides the high chromatographic performance deriving from the use of the cellulose-based Chiralpak IB column and a chloroform containing "non-standard" eluent mixture, sampling procedures are facilitated by the optimized HPLC method.

The utilization of the synthetic methodology above described for the preparation of *E*-guggulsterone needed for in *in vivo* animal model studies [29], as well as for the synthesis of guggulsterone derivatives with improved potency and selectivity for the FXR receptor, is currently underway and the results will be reported in due time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.11.012.

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