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HPLC and TLC characterisation of ecdysteroid alkyl ethers

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ABSTRACT

Semi-synthetic ecdysteroid alkyl ethers have increased potential over natural ecdysteroids as actuators of ligand-inducible gene-expression systems based on the ecdysteroid receptor for *in vivo* applications. However, a scalable synthesis of these compounds has yet to be developed. We report a set of reversed-phase (RP; C_{18} and C_6) and normal-phase (NP; diol) HPLC systems which can be used to analyse and separate ecdysteroid ethers with single or multiple *O*-methyl substitutions at the 2α-, 3β-, 14α-, 22- and 25-positions. The elution order of methyl ether analogues of the prototypical ecdysteroid 20-hydroxyecdysone (20E) was 3-methyl < 2-methyl < 14-methyl < 25-methyl < 22-methyl with both C_{18}- and C_6-RP-HPLC, when eluted with methanol/water mixtures. Further, the elution order of 20E 22-*O*-alkyl ethers was methyl < ethyl < allyl < *n*-propyl < benzyl < *n*-butyl with both C_{18}- and C_6-RP-HPLC. Moreover, the ecdysteroid alkyl ethers can also be adequately resolved by NP-HPLC and silica HPTLC. On the latter, detection of ecdysteroid *O*-alkyl ethers with the *p*-anisaldehyde/sulphuric acid reagent distinguishes 22-*O*-alkyl ethers from non-22-*O*-alkyl ether analogues by the colour of the resulting spot.

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1. Introduction

Ecdysteroids are the moulting hormones of arthropods. Their biological activity is mediated by binding to a specific transcription factor, the ecdysteroid receptor (EcR), which regulates gene-expression in target cells [1–3]. Ecdysteroid-induced, EcR-based engineered gene-expression systems are suitable technologies for the tight control of transgene expression in mammalian cells [4,5]. Since neither ecdysteroids, nor their specific receptors, are synthesised in vertebrates, they provide a particularly attractive system for the regulation of transferred exogenous genes in vertebrates. EcR-based gene switches have been successfully applied in animal models (zebrafish [6], mouse [4,7,8], rat [9]), both in functional genomics [10,11] and disease models [12,13], and are candidate systems for switch-controlled human gene therapy [14–17]. Thus far, feasibility has been demonstrated for two ecdysteroid ligands: ponasterone A (PoA **25**; Fig. 1) and muristerone A (5β,11α-dihydroxyPoA). Despite a promising pharmacology and a benign toxicological profile [18,19], natural ecdysteroids possess unoptimized pharmacokinetic properties for use as drugs in mammals. Their high degree of hydroxylation, typically at several or all of the 2-, 3-, 14-, 20-, 22- and 25-positions on the steroid skeleton (Fig. 1), infers a low tissue permeability and metabolic lability [18]. In order to overcome these drawbacks, we have recently reported novel semi-synthetic analogues of prototypical ecdysteroids, PoA and its 25-hydroxy analogue, 20-hydroxyecdysone (20E **24**), in which the hydroxyl groups have been refashioned into more suitable pharmacophore elements by alkyl-capping [20]. The alkyl ether analogues appear to possess more favourable absorption, distribution, metabolism and excretion properties with respect to their parent ecdysteroids, while retaining gene-switch potency in favourable cases [20,21]. There is a demand for scalable syntheses of such analogues for the development of novel ecdysteroid-regulated gene switches. Consequently, a simple, sensitive and reliable strategy for simultaneous determination of individual *O*-alkyl ecdysteroids in crude reaction mixtures would be highly desirable, owing to the multiple hydroxyl groups which may be alkylated.

A large structural diversity of ecdysteroids is also found in plants (phytoecdysteroids), including a variety of polar (glycoside/sulphate) and non-polar (acetate, benzoate, acetonide) conjugates [22,23]. However, natural ecdysteroid mixtures do not generally include alkyl ether analogues, as this functional group has so far been detected only in a single case, i.e. polypodoaurein (20E 25-methyl ether), a minor constituent of the fern *Polypodium*

Abbreviations: DAD, Diode Array Detector; 20E, 20-hydroxyecdysone; 20E-XOAc, derivative of 20E with acetate group(s) at C–X; 20E or PoA X–OMe, 20E or PoA derivative with methyl ether group(s) at C–X; EcR, ecdysteroid receptor; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; ODS, octadecylsilane; PoA, ponasterone A; R_f, retention factor; R_t, retention time; TLC, thin-layer chromatography.

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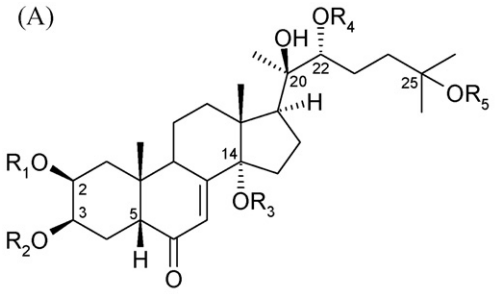

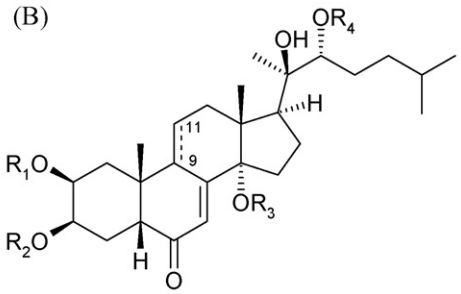

No.		Core	R1	R2	R3	R4	R5	C9-C11[a]
1	20E 2-methyl ether	A	CH_3	H	H	H	H	1
2	20E 3-methyl ether	A	H	CH_3	H	H	H	1
3	20E 14-methyl ether	A	H	H	CH_3	H	H	1
4	20E 22-methyl ether	A	H	H	H	CH_3	H	1
5	20E 25-methyl ether	A	H	H	H	H	CH_3	1
6	20E 2,22-dimethyl ether	A	CH_3	H	H	CH_3	H	1
7	20E 3,22-dimethyl ether	A	H	CH_3	H	CH_3	H	1
8	20E 14,22-dimethyl ether	A	H	H	CH_3	CH_3	H	1
9	20E 22,25-dimethyl ether	A	H	H	H	CH_3	CH_3	1
10	20E 2,3,14,22-tetramethyl ether	A	CH_3	CH_3	CH_3	CH_3	H	1
11	20E 22-ethyl ether	A	H	H	H	Et	H	1
12	20E 22-*n*-propyl ether	A	H	H	H	*n*-Pr	H	1
13	20E 22-*n*-butyl ether	A	H	H	H	*n*-Bu	H	1
14	20E 22-allyl ether	A	H	H	H	$CH_2CH{=}CH_2$	H	1
15	20E 22-benzyl ether	A	H	H	H	CH_2Ph	H	1
16	20E 22-[(2'*R*/*S*)-2'-ethyloxiran-2'-yl)] ether	A	H	H	H	C(cyclo-OCH_2)CH_2CH_3	H	1
17	PoA 2-methyl ether	B	CH_3	H	H	H	-	1
18	PoA 14-methyl ether	B	H	H	CH_3	H	-	1
19	PoA 22-methyl ether	B	H	H	H	CH_3	-	1
20	PoA 2,22-dimethyl ether	B	CH_3	H	H	CH_3	-	1
21	PoA 3,22-dimethyl ether	B	H	CH_3	H	CH_3	-	1
22	PoA 14,22-dimethyl ether	B	H	H	CH_3	CH_3	-	1
23	dacryhainansterone 22-methyl ether	B	H	H	H	CH_3	-	2
24	20-hydroxyecdysone	A	H	H	H	H	H	1
25	ponasterone A	B	H	H	H	H	-	1
26	dacryhainansterone	B	H	H	H	H	-	2

Fig. 1. Structures of ether analogues of 20-hydroxyecdysone (structure A), and ponasterone A (structure B without 9(11)-double bond) and dacryhainansterone (structure B with 9(11)-double bond). [a] 1 = single bond and 2 = double bond. Additionally, in the text and Tables, the suffix 'a' refers to the 2,3-acetonide derivative of the compound with the same number, 'b' to the 20,22-acetonide and 'c' to the 2,3;20,22-diacetonide.

aureum L. [24]. Thus, synthetic ecdysteroid ethers provide an extension to the diversity of natural analogues for biological activity studies. Owing to the previous absence of ecdysteroid alkyl ethers, it had not been possible to systematically investigate the chromatographic behaviour of this class of ecdysteroids until now. Using both reversed-phase (RP) and normal-phase (NP) HPLC systems, we analysed the chromatographic behaviour of twenty-three 20E and PoA alkyl ether analogues (**1**–**23**). In addition, preparative HPLC methods to separate individual ecdysteroids from crude alkylation mixtures are also described. In order to analyse and separate mixtures of semi-synthetic analogues with single or multiple hydroxyl group capping, we adapted HPLC methods previously developed for natural ecdysteroids [22,25–28]. TLC has also been used extensively for the separation and analysis of ecdysteroids from either plant extracts and synthetic mixtures (e.g. [29–32]); the different TLC methods applied have been recently reviewed [33]. Suitable general TLC methods involve the use of a silica gel stationary phase with a mixture of chloroform or dichloromethane/methanol, ethanol or 1-propanol in a variable ratio as mobile phase. We additionally report the chromatographic behaviour of ecdysteroid alkyl ethers on silica gel HPTLC.

2. Experimental

2.1. Materials and reagents

PoA (**25**) was supplied by Prof. René Lafont, Université Pierre et Marie Curie, Paris. 20E (**24**) was supplied by Dr. V. Volodin, Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia. The ecdysteroid ethers under study (**1**–**23**) and derivatives with the 2,3- and/or the 20,22-diol protected by acetonide groups (labelled **a**–**c**) were synthetically prepared from the parent ecdysteroids 20E and PoA, as previously described [20]. The structures and stereochemistry of **1**–**23** (summarised in Fig. 1) were unambiguously assigned by solution 1D- and 2D-NMR and high-resolution MS. Twenty-two (**1**–**22**) of them possess a typical ecdysteroid structure with a *cis*-A/B ring junction (5β-H) and a 14α-hydroxy-7-en-6-one chromophore. Methyl ether groups are present at one or more of the

2β-, 3β-, 14α-, 22-hydroxyl groups of 20E (**1**–**8**) or PoA (**17**–**22**), as well as 25-OH in 20E (**5**). Other substituents attached to the 22-OH of 20E include C_2–C_4 alkyl chains (**4**, **11**–**13**), allyl (**14**), benzyl (**15**) or 2′-ethyloxiranyl (**16**) moieties. *p*-Anisaldehyde was purchased from Sigma–Aldrich (Gillingham, UK). All other reagents and HPLC-grade solvents were purchased from Fisher Scientific (Loughborough, UK). Water for HPLC was deionised to a degree of purity of 17 Ω.

2.2. HPLC

The HPLC systems (Gilson) used were (i) two 306 pumps with an 805 manometric module and 811B dynamic mixer, controlled by a 506C system interface module, together with a 170 Diode Array Detector (DAD; monitoring at wave-lengths of 242 and 300 nm), using Unipoint Software for system control and data handling, and (ii) two 303 pumps, controlled by Gradient Manager Model 702 software, with an 802C manometric module and mixer unit with a variable wave-length Holochrome 115 UV detector set at 242 nm, connected to a Shimadzu CR3A integrator and a Philips PM8251 recorder. HPLC equipment was purchased from Anachem Ltd. (Luton, UK).

Analytical HPLC was performed with both RP- and NP-HPLC systems. The columns were C_{18} Phenomenex Sphereclone ODS2, 5 μm, 150 mm × 4.60 mm (C_{18}-RP-HPLC) and C_6 Phenomenex Sphereclone, 5 μm, 150 mm × 4.6 mm (C_6-RP-HPLC) (Phenomenex, Macclesfield, UK), and Apex II diol column, 5 μm, 150 mm × 4.6 mm (GRACE, Wilmington, DE, USA). All analytical systems were used at a flow-rate of 1 mL/min and with detection at the λ = 242 and 300 nm. For analytical RP-HPLC, the solvent system was a linear gradient of methanol/water from 30 to 100% in 25 min using C_{18}-RP-HPLC (method A) or C_6-RP-HPLC (method B). For analytical NP-HPLC, the solvent system was a linear gradient of methanol/dichloromethane from 2 to 10% (method C) or 4 to 10% (method D) in 20 min. Semi-preparative HPLC was performed with a RP-HPLC column (Phenomenex Sphereclone ODS2, 5 μm, 250 mm × 10 mm), a NP-HPLC column (GRACE Apex II diol, 5 μm, 150 mm × 8 mm) and/or a silica column (Kinesis Zorbax Sil, 5 μm, 250 mm × 9.4 mm), at a flow-rate of 2 mL/min. Preparative RP-HPLC was performed with a Phenomenex Sphereclone ODS2 column, 5 μm, 250 mm × 21.2 mm, at a flow-rate of 5 mL/min. All solvents were degassed prior to use by filtration under suction through 0.45 μm (for aqueous solutions) or 0.5 μm (for organic solutions) Waters Millipore® filters. For semi-preparative and preparative RP-HPLC, columns were eluted isocratically with appropriate (50–80%, v/v) methanol/water mixtures. The semi-preparative diol column was eluted with methanol (2%, v/v) in dichloromethane, and the silica column with dichloromethane/2-propanol/water (125:30:2 or 160:30:1.5, v/v/v).

Samples were filtered through a Minisart® 0.20 μm filter (Sartorius, Epsom, UK) prior to manual injection. For analytical HPLC, injected solutions typically contained 0.1–10 μg of each ecdysteroid (quantified by UV-spectroscopy); compounds were dissolved in 30 μL of the appropriate mobile phase and injected into a 100 μL loop.

2.3. UV spectrophotometry

The characteristic ultra-violet (UV) spectra of ecdysteroids have been described previously [34]. UV spectra of ecdysteroids were determined in methanol using a Shimadzu UV-2401 PC UV–VIS dual-beam spectrophotometer and quartz cells (Hellma, Essex, UK).

2.4. TLC

HPTLC of ecdysteroids was performed on silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) using chloroform:methanol ratios of 7:1, 10:1 or 15:1 (v/v). Samples were applied as spots along the origin of a 20 cm × 10 cm plate, 1 cm from the bottom, using a capillary tube. The plate was dipped in the appropriate solvent system in a TLC tank previously saturated with the vapour of the mobile phase. After the plate was developed, samples were visualised by UV fluorescence quenching at 254 nm, followed by dipping the plate into a 5% (w/v) *p*-anisaldehyde and 5% (v/v) sulphuric acid solution in ethanol and heating with a heat-gun until colours appeared. Mobility of compounds is expressed as retention factor (R_f) values (R_f = distance moved by compound/distance moved by solvent front).

3. Results and discussion

3.1. RP-HPLC

Analytical RP-HPLC systems were used to analyse the impact of the number and the position of the alkyl ether groups of ecdysteroids **1**–**23** on chromatographic behaviour (Table 1, methods A and B). The elution order of 20E and PoA methyl ethers depended upon the position of the ether group(s), and both mono- and dimethyl ethers followed the same sequence. As an example, the C_{18}-RP-HPLC chromatogram of a mixture of 20E and its mono- and dimethyl ether analogues is shown in Fig. 2. 20E was separated well from the *O*-alkylated analogues and, among these, 20E 22-methyl ether **4** was the most resolved. The elution sequence was 20E 3-methyl ether **2** < 20E 2-methyl ether **1** < 20E 22-methyl ether **4** < 20E 3,22-dimethyl ether **7** < 20E 2,22-di methyl ether **6**, which was maintained eluting the mixture on a C_6-RP-HPLC column under analogous HPLC conditions (Table 1). The corresponding 20E acetates showed a similar sequence order in RP-HPLC (20E 3-acyl < 20E 22-acyl < 20E 2-acyl < 20E 3,22-diacyl < 20E 2,22-diacyl) [22], with the only difference being an inverted order for 20E 22-OAc and 20E 2-OAc. However, the different mobile phases used for eluting the 20E acetates (linear gradient from 20 to 70% acetonitrile in 0.1% trifluoroacetic acid over 45 min) could account for the observed change in selectivity. On the other hand, 20E 14-methyl ether **3**, 20E 25-methyl ether **5**, and their respective 22-dimethyl

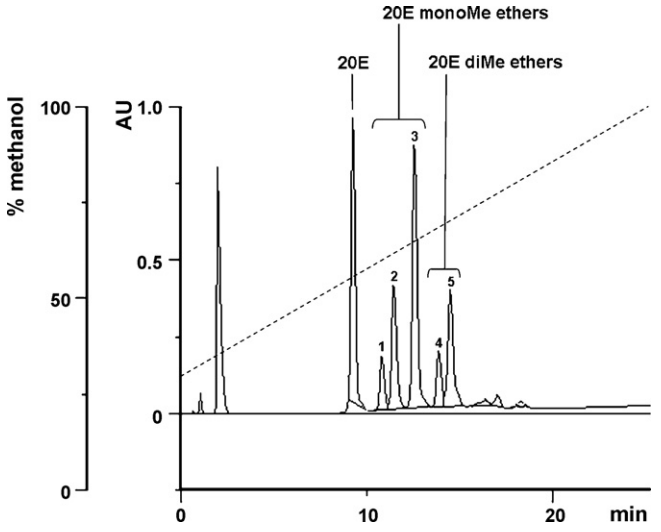

Fig. 2. HPLC/DAD chromatogram of the crude reaction mixture during *O*-methylation of 20E on an analytical C_{18}-RP-HPLC column (Section 2.2) subjected to a gradient from 30 to 100% methanol (ascending dotted line) in water in 25 min, at a flow-rate = 1 mL/min. Effluent was of monitored at λ = 242 and 300 nm. Peak assignment: 1, 20E 3-methyl ether; 2, 20E 2-methyl ether; 3, 20E 22-methyl ether; 4, 20E 3,22-dimethyl ether; 5, 20E 2,22-dimethyl ether. The large peak with R_t = 2.5 min corresponds to residual *N*,*N*-dimethylformamide from synthetic reactions. AU = absorbance units.

Table 1
Elution times of 20E and PoA alkyl ether derivatives and their acetonides in analytical HPLC systems using a flow-rate of 1 mL/min with detection wave-lengths of 242 and 300 nm. The solvent systems were a linear gradient of methanol/water from 30 to 100% in 25 min using C_{18}-RP-HPLC (method A) or C_6-RP-HPLC (method B); or the diol-NP-HPLC with a linear gradient of methanol/dichloromethane from 2 to 10% (method C) or 4 to 10% (method D) in 20 min.

No.	Ecdysteroid	RP-HPLC Method A	Method B	NP-HPLC Method C	Method D
1	20E 2-methyl ether	11.1	8.7	–	4.4
2	20E 3-methyl ether	10.6	8.4	–	4.3
3	20E 14-methyl ether	11.2	9.4	–	4.1
3a	14-Methyl ether 20E 2,3;20,22-diacetonide	24.3	–	–	–
4	20E 22-methyl ether	12.3	9.8	–	6.3
4a	2,3-Acetonide 20E 22-methyl ether	18.6	–	–	–
5	20E 25-methyl ether	11.4	9.6	–	4.4
5a	2,3-Acetonide 20E 25-methyl ether	17.8	–	–	–
6	20E 2,22-dimethyl ether	14.1	11.3	5.5	–
7	20E 3,22-dimethyl ether	13.5	10.8	5.1	–
8	20E 14,22-dimethyl ether	14.3	11.5	5.2	–
9	20E 22,25-dimethyl ether	14.7	12.3	6.6	–
9a	2,3-Acetonide 20E 22,25-dimethyl ether	20.0	–	–	–
10	20E 2,3,14,22-tetramethyl ether	17.9	15.1	2.1	–
11	20E 22-ethyl ether	14.6	11.3	–	5.1
11a	22-Ethyl ether 20E 2,3-acetonide	19.2	–	–	–
12	20E 22-*n*-propyl ether	17.5	14.2	–	4.9
13	20E 22-*n*-butyl ether	19.2	15.7	9.9	–
14	20E 22-allyl ether	15.9	12.6	10	–
15	20E 22-benzyl ether	18.5	15.3	9.8	–
16	20E 22-[(2′*R*/*S*)-2′-ethyloxiran-2′-yl)] ether	17.1	14.1	11.2	–
17	PoA 2-methyl ether	16.5	14.5	3.6	–
18	PoA 14-methyl ether	17.7	15.3	4.9	–
19	PoA 22-methyl ether	18.0	14.8	5.7	–
19a	2,3-Acetonide PoA 22-methyl ether	22.2	-	-	–
20	PoA 2,22-dimethyl ether	19.8	16.6	2.7	–
21	PoA 3,22-dimethyl ether	18.9	16.1	2.7	–
22	PoA 14,22-dimethyl ether	20.7	17.7	3.5	–
23	Dacryhainansterone 22-methyl ether	17.2	14.3	6.6	–
24	20E	9.1	7.2	–	6.6
24a	20E 2,3-acetonide	15.9	12.5	–	–
24b	20E 20,22-acetonide	19.4	–	–	–
24c	20E 2,3;20,22-diacetonide	23.0	–	–	–
25	PoA	14.7	12.0	11.1	–
25a	PoA 2,3-acetonide	20.0	–	–	–

ether analogues **8** and **9**, were incompletely resolved from other 20E *O*-methyl derivatives in both the analytical C_{18}- and C_6-RP-HPLC gradient systems; resolution can be improved by increasing the column length.

PoA mono- and dimethyl ether analogues (**17**–**22**) showed slightly greater retention times than the corresponding 20E derivatives (Table 1), but with a generally similar sequence of elution, reflecting the chromatographic behaviour of the parent ecdysteroids [26,27]. Dacryhainansterone (or $\Delta^{9,11}$-ponasterone A) 22-OMe **23** has an additional double bond between C-9 and C-11, which shifts the UV maximum (λ_{max}) from 242 nm (for the typical ecdysteroid chromophore) to 299 nm (see below). The elution time of **23** does not greatly differ from that of the corresponding monomethylated PoA derivative, as a result of the only small alteration in steroid core planarity brought about by the additional double bond.

The 20E derivatives with progressively longer (C_1–C_4) linear chains attached at the 22-oxygen (**4**, **11**–**13**) showed corresponding increases in elution time in the RP-HPLC systems applied here (Tables 1 and 2). 20E 22-allyl **14** possessed a retention time (R_t) which is intermediate between 20E 22-ethyl ether **11** and 20E 22-*n*-propyl ether **12**, owing to the unsaturated system. Likewise, owing to the aromatic system, 20E 22-benzyl ether **15** showed a shorter R_t than 22-*O*-alkyl derivatives with much smaller substituent groups, such as 20E 22-*n*-butyl ether **13**.

Semi-preparative and preparative C_{18}-RP-HPLC systems eluted with suitable isocratic solutions of methanol in water were used for separation work. Solvent systems allowing separation of individual analogues in 20E or PoA alkyl ether mixtures are reported in Table 2. In the few cases of similar retention times or co-eluting compounds, the combination of two different RP-HPLC systems allowed the complete resolution of individual components of the mixture. For example, PoA 14-methyl ether **18** and dacryhainansterone 22-methyl ether **23**, both eluting at 25 min in the preparative C_{18}-RP-HPLC system with 75% methanol in water, separated well using the semi-preparative C_{18}-RP-HPLC system with 65% methanol in water.

3.2. NP-HPLC

For analytical NP-HPLC, a linear gradient from 4 to 10% methanol/dichloromethane over 20 min was employed to separate 20E monomethyl ethers, while a 2–10% gradient was used to separate less polar compounds, including 20E derivatives with two or more methoxy groups and PoA methyl ethers (Table 1, methods C and D). However, under these conditions, resolution of 20E monomethyl analogues **1**–**5** was not extensive (R_ts in the range 4.1–4.4 min, except for 20E 22-methyl ether which had a R_t of 6.3 min). Likewise, 20E dimethyl ether analogues **6**–**9** showed similar R_ts (between 5.1 and 5.5 min, except for 20E 22,25-dimethyl ether which had a R_t of 6.6 min). A similar situation was observed for PoA derivatives **17**–**22**. This is a consequence of the high eluting power of the methanol/dichloromethane mixtures used as NP-HPLC solvent systems, resulting in very short retention times. It is expected that by changing the NP-HPLC conditions in order to obtain a R_t of ca. 20 min for 20E (i.e. using a 2% methanol/dichloromethane isocratic solution on a semi-preparative diol-bonded phase, cf. Table 2), the efficiency of

Table 2
Elution times of 20E and PoA alkyl ether derivatives and their acetonides on semi-preparative HPLC (at a flow-rate of 2 mL/min) and preparative HPLC (at a flow-rate of 5 mL/min) with detection wave-lengths of 242 or 300 nm. Solvent systems are reported in brackets (methanol in water for RP-HPLC). Retention times are expressed in minutes.

No.	Ecdysteroid ether	Retention times (min)		
		Semi-prep. C_{18} RP-HPLC	Prep. C_{18} RP-HPLC	Semi-prep. diol-bonded silica NP-HPLC
1	20E 2-methyl ether	23 (50% MeOH)	–	–
2	20E 3-methyl ether	20 (50% MeOH)	–	–
3	20E 14-methyl ether	21 (50% MeOH)	–	–
3a	14-methyl ether 20E 2,3;20,22-diacetonide	33 (80% MeOH)	–	–
4	20E 22-methyl ether	17 (58% MeOH)	28 (60% MeOH)	–
4a	22-Methyl ether 20E 2,3-acetonide	23 (70% MeOH)	–	–
5	20E 25-methyl ether	–	21 (60% MeOH)	–
6	20E 2,22-dimethyl ether	24 (60% MeOH)	–	–
7	20E 3,22-dimethyl ether	21 (60% MeOH)	–	–
8	20E 14,22-dimethyl ether	22 (60% MeOH)	–	16 (CH_2Cl_2:2-PrOH:H_2O, 160:30:1.5)
9	20E 22,25-dimethyl ether	–	42 (60% MeOH)	–
10	20E 2,3,14,22-tetramethyl ether	19 (70% MeOH)	–	–
11	20E 22-ethyl ether	12 (70% MeOH)	–	20 (CH_2Cl_2:2-PrOH:H_2O, 125:30:2)
12	20E 22-*n*-propyl ether	18 (70% MeOH)	–	–
13	20E 22-*n*-butyl ether	–	33 (75% MeOH)	–
14	20E 22-allyl ether	–	26 (70% MeOH)	–
15	20E 22-benzyl ether	–	48 (70% MeOH)	–
16	20E 22-[(2′*R*/*S*)-2′-ethyloxiran-2′-yl)] ether	–	21 (75% MeOH); 30 (70% MeOH)	–
17	PoA 2-methyl ether	25 (65% MeOH)	21 (75% MeOH)	18 (2% MeOH/CH_2Cl_2)
18	PoA 14-methyl ether	32 (65% MeOH)	25 (75% MeOH)	15 (2% MeOH/CH_2Cl_2)
19	PoA 22-methyl ether	42 (65% MeOH)	29 (75% MeOH)	18 (2% MeOH/CH_2Cl_2)
20	PoA 2,22-dimethyl ether	21 (75% MeOH)	43 (75% MeOH)	–
21	PoA 3,22-dimethyl ether	47 (65% MeOH)	35 (75% MeOH)	11 (2% MeOH/CH_2Cl_2)
22	PoA 14,22-dimethyl ether	–	54 (75% MeOH)	10 (2% MeOH/CH_2Cl_2)
23	Dacryhainansterone 22-methyl ether	28 (65% MeOH)	25 (75% MeOH)	–
24	20E	–	16 (60% MeOH)	35 (CH_2Cl_2:2-PrOH:H_2O, 125:30:2)
25	PoA	–	16 (75% MeOH)	–

separation of 20E singly and multiply derivatised ether analogues will improve accordingly, while resolution within analogues with the same number of methyl groups may also improve in some cases. More complex eluting solutions, such as ternary mixtures of dichloromethane, 2-propanol and water, are also widely used for NP separation of natural ecdysteroids on silica columns [22]. Such systems were successfully applied to *O*-alkyl ether derivatives. We found that the original mixture (125:30:2, v/v/v) was suitable for the separation of ecdysteroids having one alkyl ether substituent, while the percentage of 2-propanol had to be decreased for less polar derivatives, e.g. to 160:30:1.5 (v/v/v) for disubstituted methyl ethers (data not shown). In these systems, a water content just below saturation reduced peak tailing, allowing a better resolution.

3.3. General remarks on HPLC of ecdysteroid alkyl ethers

For natural ecdysteroids, it is generally accepted that a combination of one NP- and one RP-system will allow the complete resolution of individual components of complex mixtures [26], as co-eluting compounds during RP-analysis are usually resolved on a NP-system [35]. In our experience, this criterion is valid also for mixtures of 20E and PoA ecdysteroid methyl ethers. Moreover, in the case of semi-preparative or preparative systems, just one or occasionally two C_{18}-RP-HPLC systems are sufficient for separation, providing suitable isocratic conditions are used which depend primarily on the number and position of the methyl substituents.

In many cases when analysing samples from organic synthesis reactions it is profitable to obtain UV spectra of the different peaks in order to characterise each entity of the chromatogram by its UV-absorbance. In addition to retention time and co-elution with a reference compound, the UV spectrum represents a supplementary piece of evidence for compound identity and purity. Most ecdysteroids possess a characteristic strong UV-absorbance at a wave-length (λ) of 242 nm in methanol or ethanol solution (extinction coefficient $\varepsilon = 12{,}400\ L\ mol^{-1}\ cm^{-1}$ [36]), which results from the presence of the 14α-hydroxy-7-en-6-one moiety, allowing detection of these steroids in the low nanogram range [22]. The relative composition of a UV-absorbing mixture may be quantified by measuring the area of selected peaks in relationship to the total area absorbing at 242 nm.

Products bearing alterations of the typical chromophore can be detected by monitoring the sample for maximal absorbance at wave-lengths other than 242 nm. For example, dacryhainansterone 22-*O*-methyl ether **23**, a compound generated during methylation of PoA, shows a $\lambda_{max} = 299$ nm. The observed λ_{max} value results from the presence of the 14α-hydroxy-7,9(11)-dien-6-one conjugated system, as confirmed by structural assignment by NMR [20]. We measured the extinction coefficient of **23** by recording its ultra-violet spectrum in methanol and found an ε-value at 299 nm = $14{,}190\ L\ mol^{-1}\ cm^{-1}$. Thus, HPLC with DAD allows mixtures of *O*-alkyl ecdysteroids to be evaluated qualitatively and quantitatively, and is useful for ecdysteroid *O*-alkylation reaction monitoring.

3.4. TLC

In the analysis of the different 20E ether analogues, we found that HPTLC on silica gel with chloroform/methanol 7:1 (v/v) worked relatively well in separating the different monomethyl ether analogues (**1**–**5**) and the various dimethyl ether analogues (**6**–**9**) (Table 3). With this system, the lowest resolution occurred between the 2- and 14-monomethyl ether analogues ($\Delta R_f = 0.02$) and the 2,22- and 14,22-dimethyl ether analogues ($\Delta R_f = 0.02$); on the other hand, the analogues with a 25-*O*-methyl groups (**5**, **9**) were well resolved from those methylated at other hydroxyl positions. Chloroform/methanol solvent systems with a lower methanol content, such as 10:1 or 15:1 (v/v), were more suitable for 20E or its ether analogues with the 2,3- and/or the 20,22-diol protected by acetonide groups. By fluorescence quenching

Table 3
R_f values of 20E and its ether and acetonide derivatives.

No.	Steroid	R_f values	Colour
1	20E 2-methyl ether	0.37[a]	Purple, then olive green
2	20E 3-methyl ether	0.33[a]	Purple, then olive green
3	20E 14-methyl ether	0.39[a]	Purple, then olive green
4	20E 22-methyl ether	0.43[a]	Purple, then deep blue
5	20E 25-methyl ether	0.64[a]	Purple, then olive green
5a	20E 25-methyl ether 2,3-acetonide	0.44[b]	Purple, then olive green
6	20E 2,22-dimethyl ether	0.47[a]	Purple, then deep blue
7	20E 3,22-dimethyl ether	0.42[a]	Purple, then deep blue
8	20E 14,22-dimethyl ether	0.49[a]	Purple, then deep blue
9	20E 22,25-dimethyl ether	0.81[a]	Purple, then deep blue
9a	20E 22,25-dimethyl ether 2,3-acetonide	0.67[b]	Purple, then deep blue
14a	20E 22-allyl ether 2,3-acetonide	0.33[b]	Purple, then deep blue
15a	20E 22-benzyl ether 2,3-acetonide	0.36[b]	Purple, then deep blue
24	20E	0.28[a]	Purple, then olive green
24a	20E 2,3-acetonide	0.25[b]	Purple, then olive green
24b	20E 20,22-acetonide	0.16[b]; 0.13[c]	Purple, then olive green
24c	20E 2,3;20,22-diacetonide	0.35[c]	Purple, then olive green

Silica-gel HPTLC plate with chloroform:methanol mobile-phase systems: [a]7:1 (v/v), [b]10:1 (v/v), [c]15:1 (v/v). Colours were produced by developing the plate with *p*-anisaldehyde/sulphuric acid reagent and heating. R_f for 20E is too low with the 10:1 or 15:1 systems, and was not recorded.

at 254 nm, ecdysteroid ether analogues appeared as violet spots, like the parent compounds with free hydroxyl groups. The *p*-anisaldehyde/sulphuric acid reagent differentiated 22-alkyl ethers from ecdysteroids with a free 22-OH. At first, all ecdysteroids appeared as bright purple spots. After ca. 5 s of heating, spots corresponding to ecdysteroids with a free 22-OH turned olive green, while those corresponding to 22-alkyl ether analogues turned deep blue. The colours gradually faded as the plate cools, and after 12 h, spots initially green appear yellow-brown, and spots initially blue appeared grey-green. Although colour reactions on TLC are not in themselves adequate for compound identification, they do, in conjunction with R_f values, provide good initial indicators of compound identification. 20E and PoA ether analogues showed a similar TLC behaviour (e.g. R_f values of 20E and its monomethyl ethers: 20E < 20E 3-methyl ether < 20E 2-methyl ether < 20E 14-methyl ether < 20E 22-methyl ether < 20E 25-methyl ether). Interestingly, like the 20E 22-ethers, 20E 22-acetates are also distinguishable from the corresponding analogues with a free 22-OH by the colour of the spot (dark-green vs. olive green, respectively) they give after spraying with the vanillin–sulphuric acid reagent [34,37].

4. Conclusions

Crude synthetic mixtures may contain many wanted or unwanted products, with some of the desired products in very small amount. Ability to detect individual components of the mixture, even if present in a small amount, at different times during the course of the reaction with an amenable analytical method may greatly contribute to the success of the synthesis. HPLC methods are often suitable for this task. In this study, we examined the chromatographic behaviour of twenty-three closely related ecdysteroid analogues generated by semi-synthesis. HPLC with DAD proved a reliable technique for the analysis of the crude reaction mixture of ecdysteroid ether formation, allowing one to distinguish individual *O*-methylated ecdysteroids differing in the number of methyl groups, or just in the position of a single methyl substituent, and to separate these from other alkyl ether derivatives. Moreover, some alteration of the ecdysteroid 14α-hydroxy-7-en-6-one chromophore occurring during the chemical transformations can be easily detected on the basis of changes of the maximum absorbance values. The reported HPLC and HPTLC data can be used for the convenient reaction monitoring, separation and purity verification of ecdysteroid alkyl ether analogues.

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