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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.

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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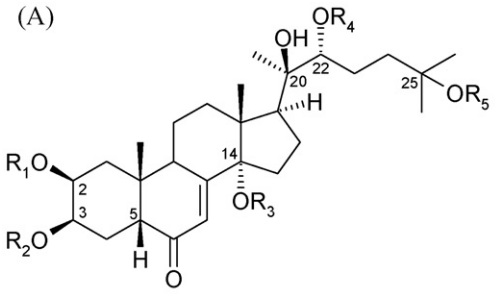

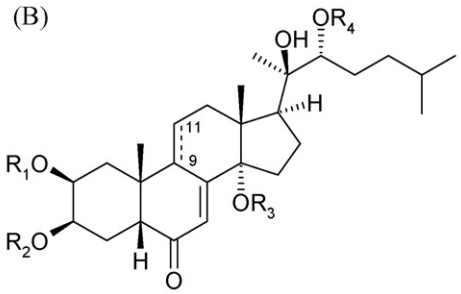

No.		Core	R1	R2	R3	R4	R5	C9-C11[a]
1	20E 2-methyl ether	A	CH_3	H	H	H	H	1
2	20E 3-methyl ether	A	H	CH_3	H	H	H	1
3	20E 14-methyl ether	A	H	H	CH_3	H	H	1
4	20E 22-methyl ether	A	H	H	H	CH_3	H	1
5	20E 25-methyl ether	A	H	H	H	H	CH_3	1
6	20E 2,22-dimethyl ether	A	CH_3	H	H	CH_3	H	1
7	20E 3,22-dimethyl ether	A	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