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Hydroxysteroid dehydrogenase transformations of 5β-scymnol and identification of oxoscymnol transformation products by liquid chromatography–tandem mass spectroscopy

Linda L. Glowacki[a], Lynn D. Hodges[a], Paul M. Wynne[b], Nicolette Kalafatis[a], Paul F.A. Wright[c], Theodore A. Macrides[a,*]

[a] Natural Products Research Group, School of Medical Sciences, RMIT University, Victoria, Australia
[b] Shimadzu Scientific Instruments (Oceania) Pty Ltd, Victoria, Australia
[c] Toxicology Key Centre, School of Medical Sciences, RMIT University, Victoria, Australia

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ABSTRACT

A new and sensitive high performance liquid chromatography (HPLC) separation procedure coupled with tandem mass spectroscopy (MS and MS^2) detection was developed to identify for the first time the oxidation products of 5β-scymnol [(24R)-(+)-5β-cholestan-3α,7α,12α,24,26,27-hexol] catalysed by bacterial hydroxysteroid dehydrogenase (HSD) reactions *in vitro*. The authentic scymnol (MW 468) standard yielded a protonated molecular ion $[M+H]^+$ at *m/z* 469 Da, and higher mass adduct ions attributed to $[M+NH_4]^+$ (*m/z* 486), $[M+H+CH_3OH]^+$ (*m/z* 501) and $[M+H+CH_3COOH]^+$ (*m/z* 530). (24R)-(+)-5β-Cholestan-3-one-7α,12α,24,26,27-pentol (3-oxoscymnol, *m/z* 467 Da, relative retention time (RRT) = 0.89) was identified as the principle molecular species of scymnol in the reaction with 3α-HSD pure enzyme. $[S]_{0.5}$ for the reaction of 3α-HSD with scymnol as substrate was 0.7292 mM. (24R)-(+)-5β-cholestan-7-one-3α,12α,24,26,27-pentol (7-oxoscymnol, *m/z* 467 Da, RRT = 0.79) and (24R)-(+)-5β-cholestan-12-one-3α,7α,24,26,27-pentol (12-oxoscymnol, *m/z* 467 Da, RRT = 0.81) were similarly identified as principle molecular species in the respective 7α-HSD and 12α-HSD reactions. Polarity of the oxoscymnol species was established as 7-oxoscymnol > 12-oxoscymnol > 3-oxoscymnol > scymnol (in order from most polar to least polar). Confirmation that 5β-scymnol is an oxidative substrate for steroid-metabolising enzymes was made possible by the use of sophisticated liquid chromatography–mass spectrometry (LC–MS) techniques that will likely provide the basis for further exploration of scymnol as a therapeutic compound.

1. Introduction

5β-Scymnol (Fig. 1) is a naturally occurring elasmobranch bile alcohol [1] that is protective against the liver toxicities of α-amanitin [2], carbon tetrachloride [3] and paracetamol [4,5] in experimental rodents. However, the xenobiotic metabolism of 5β-scymnol in these and other mammals including humans is unknown. The lack of a suitable analytical method to detect and identify scymnol transformation products in biological samples [6] has severely hampered its *in vivo* investigation.

Akin to the 24-carbon bile acids of the higher vertebrates, the 27-carbon bile alcohols of the elasmobranch are saturated, hydroxylated sterols based on the cyclopentanophenanthrene skeleton [7]. Having been synthesised in hepatocytes and secreted into the bile as end products of cholesterol metabolism, bile sterols are shown to undergo enzyme-catalysed biotransformations, both within the liver [8] and during their enterohepatic cycling [9], that involve steroid ring system and side chain modifications. Invariably, bile sterols undergo conjugation reactions at side-chain termini, which serve to increase their hydrophilicity and decrease their membrane permeability characteristics [10]. Bile acid conjugation is by amidation with taurine (major) or glycine (minor), whereas bile alcohol conjugation is by esterification with sulfate. 5β-Scymnol is a unique 27-carbon bile sterol in that the terminal methyl groups C26 and C27, together with the chiral C24 are present as alcohols. In the sharks and stingrays of the elasmobranch, the scymnol side chain undergoes sulfation by hepatic sulfotransferases at either the C26 or C27 position, but not both [11]. The two possible diastereoisomeric forms, deduced from the chemical shifts of the carbon nuclei by ^{13}C nuclear assignment of 5β-scymnol sulfate [12], have the configurations C24R, C25R and

* Corresponding author at: School of Medical Sciences, RMIT University, PO Box 71, Bundoora 3083, Victoria, Australia. Tel.: +61 3 9925 7070; fax: +61 3 9925 7063.
E-mail address: macrides@rmit.edu.au (T.A. Macrides).

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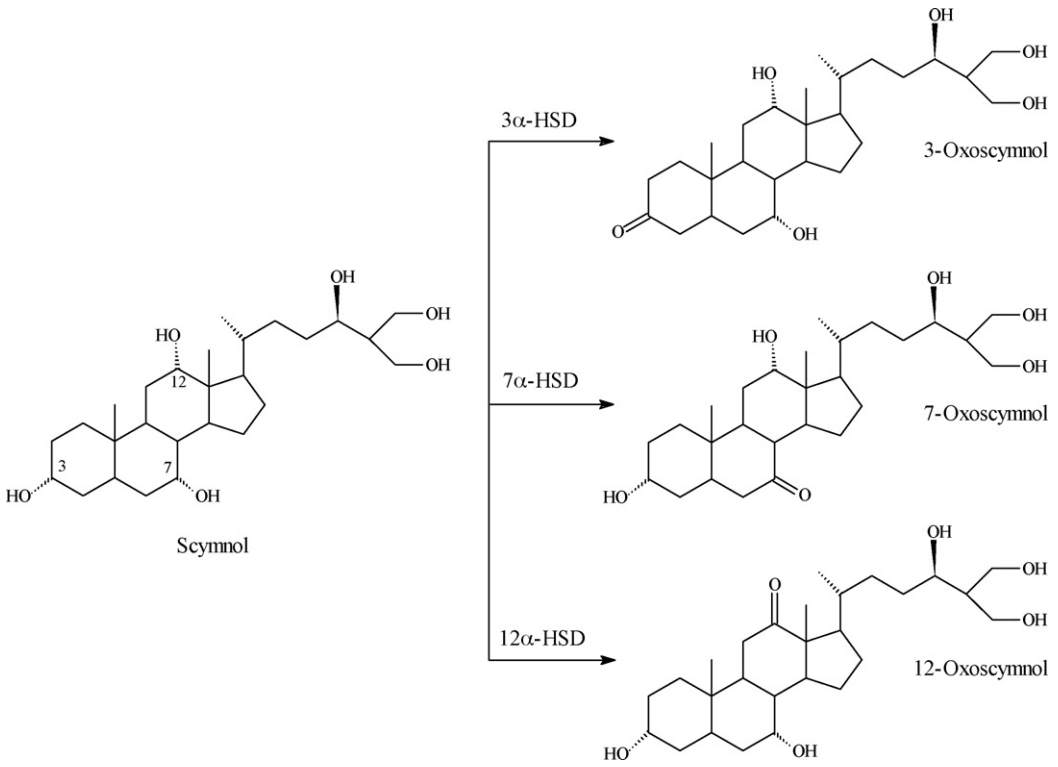

Fig. 1. Structural formula for 5β-scymnol as a substrate for bacterial 3α-, 7α- and 12α-hydroxysteroid dehydrogenase (HSD) enzymes.

C24R, C25S, respectively. There is growing support for the utility of the alcohol-substituted aliphatic side chain of scymnol as a functioning antioxidant [4,5,13] and free radical scavenger [14,15] in hepatoprotective mechanisms.

Chromatographic methods for bile sterol analysis employ gas chromatography and high performance liquid chromatography (HPLC) separation coupled with a suitable detection system [16]. In the analysis of scymnol by HPLC, refractive index (RI) and evaporative light-scattering (ELS) detection are applicable for biological samples such as crude shark bile [13]. In contrast to RI which requires isocratic elution conditions, ELS is independent of mobile phase changes and able to analyse free and sulfated scymnol in the same chromatographic run. A recent development for scymnol analysis by HPLC incorporates the use of an in-line post-column immobilised enzyme detection system [17]. The method is specific for 3α-hydroxy bile sterols such as scymnol, but fails to detect those lacking the α-hydroxyl group at this position. Accordingly, alternative detection with enhanced sensitivity and selectivity that is suitable for HPLC analysis of scymnol and modified scymnol compounds is required. The method of liquid chromatography–mass spectrometry (LC–MS) [16], which has not been previously used to analyse scymnol, offers an alternative to the existing analytical methods.

This paper describes for the first time a highly sensitive HPLC separation coupled with tandem MS detection for the isolation and structural identification of scymnol and hydroxysteroid dehydrogenase (HSD)-generated scymnol transformation products. Hydroxysteroid dehydrogenase reactions are fundamental to the *in vivo* transformations of bile sterols, catalysing regio-specific oxidations of hydroxyl groups at steroidal carbons C3, C7 and C12 [9]. The presence of 3α-, 7α- and 12α-hydroxyls on scymnol is compelling in support of a *prima facie* investigation of scymnol oxidation products in the HSD reactions.

2. Experimental

2.1. Chemicals

5β-Scymnol was supplied by McFarlane Marketing (Aust.) Pty Ltd. (Melbourne, VIC, Australia). 3α-Hydroxysteroid dehydrogenase (3α-HSD, EC1.1.1.50, from *Pseudomonas testosteroni* (*Comamonas testosteroni*)), 7α-hydroxysteroid dehydrogenase (7α-HSD, EC1.1.1.159, from *Escherichia coli*) and 12α-hydroxysteroid dehydrogenase (12α-HSD, EC1.1.1.176, from *Bacillus sphaericus*) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of the highest grade and purchased from local commercial sources.

2.2. HSD enzyme incubations

Standard solutions of scymnol were prepared in methanol for each assay. Enzyme incubations were performed in triplicate at 25 °C, and contained scymnol at concentrations 0.2–1.0 mM with 2.5 mM NAD^+ in 0.1 M sodium pyrophosphate buffered to pH 8.9. Each reaction was started by the addition of 50 μL of enzyme (either 3α-HSD, 7α-HSD or 12α-HSD, 0.5 units mL^{-1} in 0.1 M sodium pyrophosphate buffer, pH 8.9) to give a final enzyme concentration of 0.025 units mL^{-1} (final reaction volume = 1 mL), and the time course change in absorbance for NADH production (340 nm, extinction coefficient = 6220 M^{-1} cm^{-1}) was recorded over a period of 5 min. The reaction was stopped by the addition of ice-cold methanol (4 mL), and precipitated protein was removed by centrifugation (1000 × *g* for 10 min). The supernatant liquid was evaporated under a gentle stream of nitrogen, and the sample was reconstituted in methanol (4 mL) and stored for no longer than 24 h at −20 °C, prior to analysis by LC–MS.