

## An Introduction to Spiral Steroids

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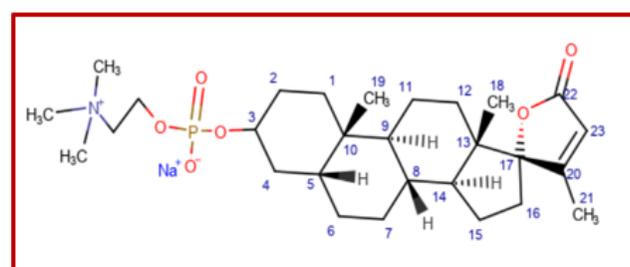
**Keywords:** DLM; Ionotropin; Spiral Steroids; Orphan Ligands; Phosphocholine Steroid Conjugates

### Abbreviations

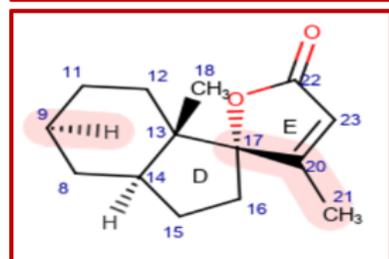
DLM: Digoxin-Like Materials; RIA: Radioimmunoassay; K<sup>+</sup>: Potassium; Na<sup>+</sup>: Sodium; MBG: Marinobufagenin; NMR: Nuclear Magnetic Resonance; MS: Mass Spectrometry; PC: Phosphocholine; DHT: 5 $\alpha$ -Dihydrotestosterone

### An introduction to spiral steroids

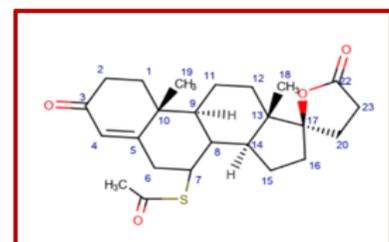
**What are spiral steroids:** Spiral steroids have several unusual structural features: two of the four valences of carbon 17 form part of Ring D while the other two valences form Ring E (See figure 1). Thus, carbon 17 is part of both rings. None of the common endogenous steroids have a spiral structure. The four rings (A, B, C and D) of most steroids form a plane (more or less) with axial methyl groups sticking up. In contrast, although Ring E is also planar, the plane is perpendicular to ABCD plane. Finally, when the  $\Delta$ 5-6 alkene is reduced, the stereochemistry must be 5 $\beta$ . In contrast, when testosterone is reduced, the product is 5 $\alpha$ -dihydrotestosterone (DHT). The enzyme that catalyzes the reduction requires the substrate to be a  $\Delta$ 4-3-ketone. The spiral steroids are all 3-phosphoesters and would not be substrates for the 5 $\alpha$ -reductase. A separate reductase enzyme is used for synthesis of bile acids such as cholic acid. This enzyme doesn't require  $\Delta$ 4-3-ketone substrates but forms 5 $\beta$ -products. This difference is significant because, despite the huge concentrations of the PC-steroids, the difference would be expected to prevent binding to the nuclear receptors for the classical steroids.



**C341 Ionotropin m/z= 546 Da**  
Carbon atoms 1-21 derive from 7-dehydrocholesterol. Carbon 22 and 23 derive from Acetyl-CoEnzyme|A



**Partial structure of Ionotropin centering on Carbon 17.** - Carbon 17 is part of both Ring D and Ring E. Ring D and Ring E are semi-planar but the planes are perpendicular. Ring E has [a] 17 $\alpha$  hydroxy group, [b]  $\beta$  spiral, and [c] a  $\gamma$ -lactone.



**Spirolactone**

**Figure 1:** Proposed structures of spiral steroids and precursors as named in this paper.

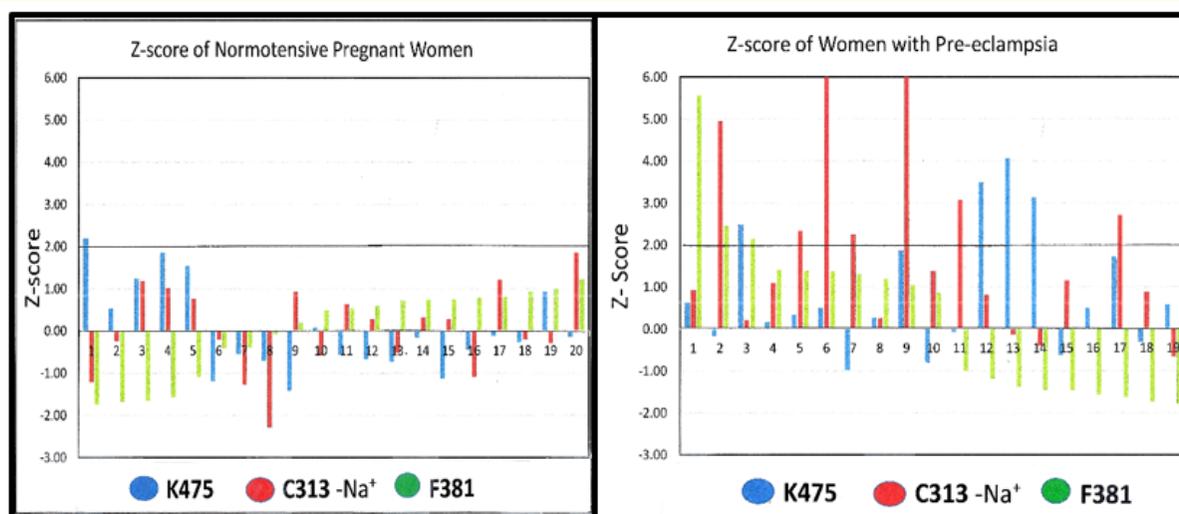
**In the beginning:** In the 18<sup>th</sup> century, Withering observed that extracts from foxglove (*Digitalis lanata*) were useful for treating congestive heart failure [1]. We now know the active component of foxglove extracts is digoxin. Digoxin is comprised of (1) tri-digitoxose, commonly called the glycone and (2) a cardiotonic steroid, commonly called the a-glycone (not the glycone). In 1955, Szent-Gyorgyi proposed that digoxin was really a substitute for an endogenous, unknown hormone [2]. However, digoxin, itself, didn't seem to be an endogenous compound because neither the glycone nor the aglycone component were naturally present in mammals. Physicians noted that it was difficult to adjust the dose for the best clinical response. The development of an RIA for digoxin led to improvements in clinical care [3]. Serum levels were generally below 0.05 ng/ml prior to therapy. Serum levels necessary for therapy had to exceed 0.6 ng/ml but levels over 2.0 ng/ml were associated with toxicity. When the digoxin assay became widely available, there were occasional reports of 'digoxin' in serum prior to therapy. In fact, one nurse was accused of murder because an infant under her care had toxic levels of 'digoxin' [4]. Later, the false assay result was attributed to an unknown digoxin-like material (DLM), perhaps just as Szent-Gyorgyi had predicted. In the last 30 years, there have been more than 300 reports (including seven from my laboratory) of an unknown DLM in patients with hypertension, pre-eclampsia, renal failure and other disorders of electrolyte regulation [5,6].

**The first candidate:** The isolation of DLM was a difficult problem because its concentration was less than 0.05 ng/ml in serum from normal patients while large volumes of serum were not available from those patients who had elevated levels. In 1991, Hamlyn reported the isolation of 13 µg of DLM from 80 liters of normal human serum [7]. They suggested the DLM was actually ouabain (g-strophanthin). Ouabain has two domains - L-rhamnose, a glycone - and a cardiotonic steroid, ouabagenin, the aglycone. Rhamnose has never been isolated from mammals. The C D spectra did not match authentic ouabain. These investigators have not confirmed the biosynthesis of either component in mammals. Further, ultra-sensitive LC-MS technology failed to detect as little as 0.002 ng/ml of ouabain in human serum [8]. Despite these discrepancies, several laboratories continue to publish about endogenous ouabain and its effect in human physiology [9]. Other scientists claim endogenous ouabain is fantasy [10,11]. Finally, when the Δ-5-6 alkene is reduced, the stereochemistry must be 5β. In contrast, when testosterone is reduced.

**The second candidate:** Recently, there has been a second candidate for the DLM-marinobufagenin (MBG). MBG is isolated by methanolic extraction of toad skin. A special antibody was developed that discriminated MBG from both ouabain and digoxin [12]. The only way that MBG has been identified in mammals is by cross-reaction with their special antibody. MBG has never been isolated and characterized from any mammalian source.

**The new candidate:** My laboratory has a new candidate for the DLM [13]. Our first candidate is/was the phosphocholine (PC) ester of a spiral steroid with 23 carbon atoms (Figure 1) We isolated about 10 mg of our candidate, which we named *IONOTROPIN*, from 10 liters of porcine blood, equivalent to 1 mg/l or 1 µg/ml. Prior to these investigations, no PC steroid esters were known and no steroids with 23 carbon atoms were known. The first paper identified 4 PC steroids. The four compounds fit together to form a biosynthetic path. <sup>31</sup>P-NMR confirmed the presence of the phosphate; the fragmentation pattern confirmed the presence of the PC; a trial and error method confirmed that there was only one composition of carbon, oxygen and hydrogen that was consistent with the molecular mass determined by mass spectroscopy. Knowledge of steroid biochemistry led to the proposed structure of Iontropin as shown in figure 1. The same compounds were also isolated and purified to near homogeneity from bovine, human, and turkey serum extracts. The PC steroids cannot be isolated by the methods used to isolate endogenous ouabain. Cord serum contains compounds that inhibit the NaK-ATPase [14]. Egg yolks contain C313, the spiral steroid precursor, while the egg whites contain spiral steroids [15]. In a more recent study, when assayed by MS-MS technology, extracts from oysters also contained ions identical to the ions present in mammals and birds. Thus, the PC steroids are not limited to mammals but are widespread throughout the animal kingdoms. The best explanation for their structure is a spiral steroid, similar to the E-ring of spironolactone (See figure 2).

**Assay by mass spectroscopy:** We have now developed a technique to measure the spiral steroids by MS in individual serum samples [16]. The first study ( $n = 40$ ) investigated samples from normotensive pregnant women ( $n = 20$ ) and women with pre-eclampsia ( $n = 20$ ). The peak intensities from the normotensive women were used to generate statistical parameters. These parameters were then used to calculate z-scores for each of the ions in the women ( $n = 20$ ) with pre-eclampsia. The z-scores are shown in figure. 12 of the 20 samples from women with pre-eclampsia had  $z \geq 2$  detected at  $m/z = 518$  Da (C313) while only 1 of the 20 samples from normotensive women had  $z \geq 2$ . This difference is statistically different at the  $P < 0.05$  level. C313 is the precursor for several spiral steroids (shown in Fig.3). Three of the samples from the women with pre-eclampsia also had high levels of ion  $m/z = 475$  Da (C329). At the time the data were as prepared, we did not know the structure of this steroid ester. Further investigation suggests that the compound is the 11 $\beta$ -hydroxy derivative of C313. Both C313 and C329 have mass ions consistent with  $\Delta^5$ - $\Delta^7$  dialkenes. As such, they are not cholesterol metabolites. Thus, it is not surprising that, to date, there have been no papers describing radiolabeled DLM starting with labeled cholesterol.



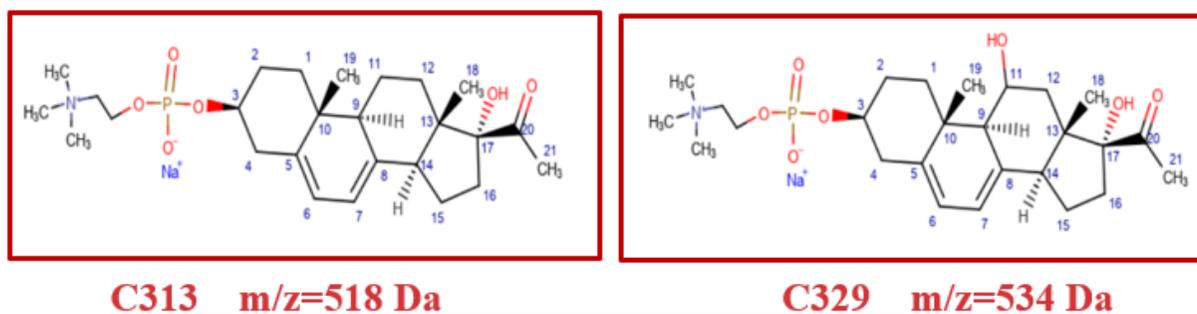
**Figure 2:** Each cluster is the Z-score of the three steroids from a single sample [16].

Color scheme [a] K475 - Blue; [b] C313 Na<sup>+</sup> cation- Red; [c] F381 - Green.

Left Panel: Normotensive pregnant women.

Right Panel: Pregnant women diagnosed with pre-eclampsia.

Positive test was defined as a Z-score over 2.



**Figure 3:** Structures of precursors for spiral steroids

**Spiral Steroids in pregnancy:** The common feature of [a] synthetic steroidal potassium sparing diuretics, [b] plant cardiotonic glycosides, and [c] the spiral steroid phosphoesters (such as Ionotropin) is the  $\gamma$ -lactone ring. Just as ethinyl estradiol and estradiol share structural features and function, we propose that the compounds sharing the lactone ring also have similar functions. Specifically, the synthetic lactones act as regulators of the NaK-ATPase, which pumps  $K^+$  into cells and  $Na^+$  out of cells, both processes must take place simultaneously for electrolyte regulation [17] and we propose that spiral steroid lactones have a similar function.

**Fetal nutrition:** The fetus gets its nutrition from the placenta. The placenta plasma electrolytes are 145 mM Na<sup>+</sup> and 4 - 6 mM K<sup>+</sup> while electrolytes in fetal tissues are 12 mM Na<sup>+</sup> and 140 mM K<sup>+</sup>. In fact, Iototropin was present in high concentration both in fetal calf serum and in human cord serum. This process is also critical during delivery [18]. After the child is born, nutrition comes from mother's milk which is high in K<sup>+</sup> [19], obviating the need to stimulate K<sup>+</sup> recovery. However, two things stand out: previously, neither the need for the transition nor its mechanism have been identified.

**Role of spiral steroids during pregnancy:** Our concept for the role of spiral steroids during pregnancy:

- The fetal placental unit synthesizes Iototropin.
- Iototropin synthesis increases during the 2<sup>nd</sup> trimester, leading to increased fetal blood pressure needed to perfuse the growing fetus (similar to the effect of cardiotonic glycosides in adults).
- In the 2<sup>nd</sup> trimester, Iototropin prevents aldosterone binding at the mineralocorticoid receptor (Pseudo-hypoaldosteronism), leading to Na<sup>+</sup> wasting and formation of amniotic fluid.
- In the 3<sup>rd</sup> trimester, high fetal concentrations of Iototropin block aldosterone synthesis (Hypoaldosteronism).
- During the immediate neonatal period, infant nutrition switches to mother's milk - 100 mM K<sup>+</sup> and 10-20 mM Na<sup>+</sup> [19]. There is no need for K<sup>+</sup> saving. The fetal-placental unit is no longer intact and Iototropin synthesis decreases to adult levels.
- After childbirth, Na<sup>+</sup> wasting continues until the Iototropin is metabolized. This physiology accounts for the known loss of fluids that occurs during the first two weeks after parturition.
- Growth resumes when the Iototropin-dependent Na<sup>+</sup> wasting ends. Overall, this process was known, but the underlying physiology was not known [20].

**Nuclear receptors:** The concentration of Iototropin in cord serum is more than 1 mg/L (> 2 μM). Most other steroids [a] are present in serum at much lower concentration, [b] are secreted episodically and [c] function by interacting with high affinity nuclear receptors. Iototropin differs on all three properties. Thus, it is likely that Iototropin functions by novel, as yet unidentified, mechanisms.

**Other target tissues:** Looking forward, spiral steroid function is not limited to pregnancy but also seems to be involved in heart, kidney, and gonadal function. We have also detected spiral steroids with 24 and 25 carbon atoms. Similar spiral steroid phosphoesters were present in oysters, indicating that the class of compounds is not limited to mammals and birds. Of greatest importance, it is clear that endogenous cardiotonic glycosides, such as endogenous ouabain, is fantasy. Both the glycone and the α-glycone are not present in mammals. We now need to reexamine the role of DLM and determine which particular spiral steroid is involved in each process. The last classical steroid discovered was aldosterone in the 1950s and 60 years later, we still have more to learn about its physiology. Our understanding of the role of the spiral steroids is just starting.

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