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Research Article

Phosphocholine Steroid Esters in Pacific Oysters (*Crassostrea gigas*)

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Abstract

Spiral steroid phosphoesters have only been reported in mammals and birds. We now wish to extend the distribution to oysters, a cold-blooded invertebrate. Based on that observation, we speculate that these compounds are probably used for some universal, hormonal function.

Freshly shucked Pacific oysters were extracted with acetonitrile, centrifuged to remove denatured proteins and filtered. The filtrate was analyzed by tandem mass spectroscopy. Spectra were obtained for the range 105 to 900 Da. In the 2nd phase of the analysis, individual ions were collected in the ion trap and were further fragmented. In total, we were able to identify peak patterns consistent with 5 novel phosphocholine steroid esters. One of the compounds was a spiral steroid (S = 365 Da); three (S = 359, 385, and 399 Da) were consistent with logical precursors for spiral steroids. In total, 31 of the 36 ions expected from the 9 compounds were present in the mass spectrum range of 450 to 620 Da. There were only five peaks in the range that could not be attributed to a steroid phosphocholine ester. The conclusion is that phosphocholine steroid esters are not limited to birds and mammals and may serve as cardiotonic steroid hormones in most species.

Based on the similarity in structure to spironolactone and the tissues in which we found high levels, we propose that one of the functions of spiral steroids is that of a potassium sparing hormone.

Keywords: *Spiral Steroids; Steroid Phosphocholine Esters; DLM; Ionotropin; Lactones*

Abbreviations

RI: Relative Ion Intensity; Da: Daltons; S: Mass Spectrum Fragment from a Steroid

Introduction

In 2018, we reported the initial discovery of a new type of steroid conjugate [1]. The compounds were phosphocholine esters and were unique in that the steroid conjugates were not estrogens, androgens, progestogens or glucocorticoids. The original report described steroid compounds that have 23 carbon atoms. There were no previously known mammalian steroids with 23 carbon atoms. Overall, we

have purified to > 95% homogeneity five of the phosphoester compounds with 23 carbon atoms. For each one, a trial and error analysis leads to one and only one composition. The chemical composition does not eliminate the possibility of multiple isomers, but a review of enzyme stereospecificity suggests unique structures. If that is not correct, then there must be additional enzymes that have not yet been identified. For example, the precursor seems to be a 17α-hydroxy-pregnenolone derivative. We can't eliminate the existence of 17β-hydroxy pregnenolone derivatives but an enzyme that makes a hydroxy derivative with that specificity has never been identified. 17β-androgens are known, but they are synthesized by reduction of 17-keto androgens, which process can't lead to pregnenolone-like compounds.

In addition to the number of carbon atoms in the steroid, the steroids are also unique in being spiral steroid lactones [2]. A spiral steroid has a carbon atom that forms part of two rings (Figure 1). As part of Ring D, carbon 17 is bound to carbon-16 and carbon-13. If the other two valences are also joined in a multi-atom ring, which is usually designated as ring E, then it would be classified as a spiral steroid. One of the other two valences is bound to carbon 20 and the fourth valence is an oxygen atom. The nomenclature is a bit confusing because the normal stereochemistry of the C-17-C-20 bond would be considered as an α-spiral steroid but the normal stereochemistry of the C-17-O bond is considered as the 17α-hydroxy group. Thus, for example, spironolactone has a 17β-hydroxy group but is an α-spiral steroid; an endogenous spiral lactone with a 17α-hydroxy group is a β-spiral steroid. Ring E could also be considered as a γ lactone [3]. Thus, a spiral steroid can be α, β, or γ depending on which aspect of the structure one considers.

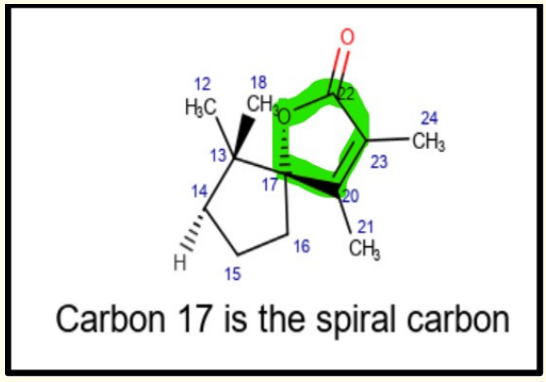

Figure 1: *Steroid fragment highlighting the spiral lactone.*

There are three things to note in this figure. [1] Carbon 17 is a β-spiral steroid; [2] the atoms highlighted in green form a γ-lactone ring; [3] A methyl group is attached to carbon 23. Thus, this fragment derives from a steroid with 24 carbon atoms. It would be formed by condensation with propionic -CoA with either C313 or C329 and subsequent ring closure.

The first paper on phosphocholine steroids described compounds from humans, pigs and cattle [1]. Until now, the only source of spiral steroid phosphoesters was in warm blooded vertebrates. Table 1 provides a summary of the phosphocholine steroids that we identified. This study was intended to determine if these compounds, or similar compounds, were also present in invertebrates and, in particular, in a species that lived in the sea. In fact, in addition to some of the same compounds we found in mammals, we found several novel compounds in the oyster extracts.

Symbol	Carbon atoms	M.W. (Da)	Composition	Possible structure elements'
C299@	21	316	$C_{21}H_{32}0_2$	Pregn-5-en-313-o1-20-one or Pregna-5,7-diene-30, 20-diol
C313&@	21#	330	$C_{21}H_{30}0_3$	Pregna-5,7-dien-30,17a-dio1-20 one
C337&	23#	354	$C_{23}H_{30}0_3$	Spiral steroid; 5, 7, 20 tri-alkene
C339&	23#	356	$C_{23}H_{32}0_3$	Spiral steroid; 5, 20 di-alkene
C359*@	23	374	$C_{23}H_{34}0_4$	Open E-ring; 5, 7 dialkene
C365*@	24	382	$C_{24}H_{30}0_4$	Spiral steroid; 5, 7, 20 tri-alkene-11-keto-23-methyl
C385*@	24	400	$C_{24}H_{32}0_5$	Open E-ring; 5, 7, 20 tri-alkene-11-keto-23-methyl
C399*@	25	414	$C_{25}H_{34}0_5$	Open E-ring; 5, 7, 20 tri-alkene -23 acetoacetyl

Table 1: *Symbols and proposed chemical composition of steroid phosphocholine esters.*

**: Open ring compounds have m/z values 2 Da higher than predicted based on their composition.*

#: Compounds that have been purified to > 90% homogeneity.

&: Compounds found in mammals.

@: Compounds found in oysters.

This table lists the symbols and the proposed chemical composition of the phosphocholine steroids we detected by mass spectroscopy. To date, we have identified in excess of 20 phosphoester steroids. Compounds that seem to be precursors are not included on the list. The molecular composition is restricted by the chemistry rules but it doesn't eliminate other possible isomers.

Methods

Freshly "shucked" oysters were homogenized in a home-style blender and the tissue was extracted with acetonitrile (3:1 - v/v). Insoluble material was removed by centrifugation. The extract was filtered in two steps: [1] with Whatman #1 filter paper and [2] Whatman syringe filters of 0.2 µm pore size. The extract was analyzed by direct injection, via the electrospray source, into the mass spectrometer. Flow rate was 10 µl/min. The capillary temperature was 275°C. Spray voltage was 4.4 volts. Ten mass spectra scans were collected, averaged and printed. Each sample was also analyzed when 60 volt was applied at the source to fragment the molecules. The identity of representative peaks was also confirmed with MS^N methodology. Between samples, to restrict cross contamination, the capillaries were washed with 4:1 acetonitrile/water until the ion counts dropped to below 1 x 10^3, i.e. less than 1% of the intensity observed in the samples being investigated. All spectra were generated in the positive ion mode.

Results

Figure 2 shows the mass spectrum obtained on the oyster extract. Although ^{12}C atoms have a mass of 12.00 Da, ^{13}C makes up about 1% of the natural carbon isotopes. As there are between 21 and 25 carbon atoms in each steroid phosphocholine ester, there will be a 'shadow' ion with a relative intensity (RI) in a similar range, i.e. 1 Da larger and with an RI of about 21 - 25% of the parent ion. 17α-Hydroxy-pregna-5,7-dienolone, the smallest molecule that forms a spiral steroid, has a molecular mass of 330 Da. There were 9 ion fragments between 300 Da and 420 Da that could derive either from steroid (S) phosphocholine conjugates or from unrelated compounds, where S is the fragment m/z actually observed without the 3-hydroxy group. S-Phosphocholine esters generate a fragmentation pattern of m/z ions (1):

The B fragment derives from the H^+ ion form of the intact phosphocholine ester. The C fragment derives from the Na^+ ion. The A fragment derives from the C fragment by loss of the trimethylamine (59 Da). The D fragment is the K^+ ion. Figure 3 shows the A, B, C, and D ions derived from C365 with its S fragment at m/z = 365 Da. The mass of the parent ion of this S fragment would be m/z = 382 Da.

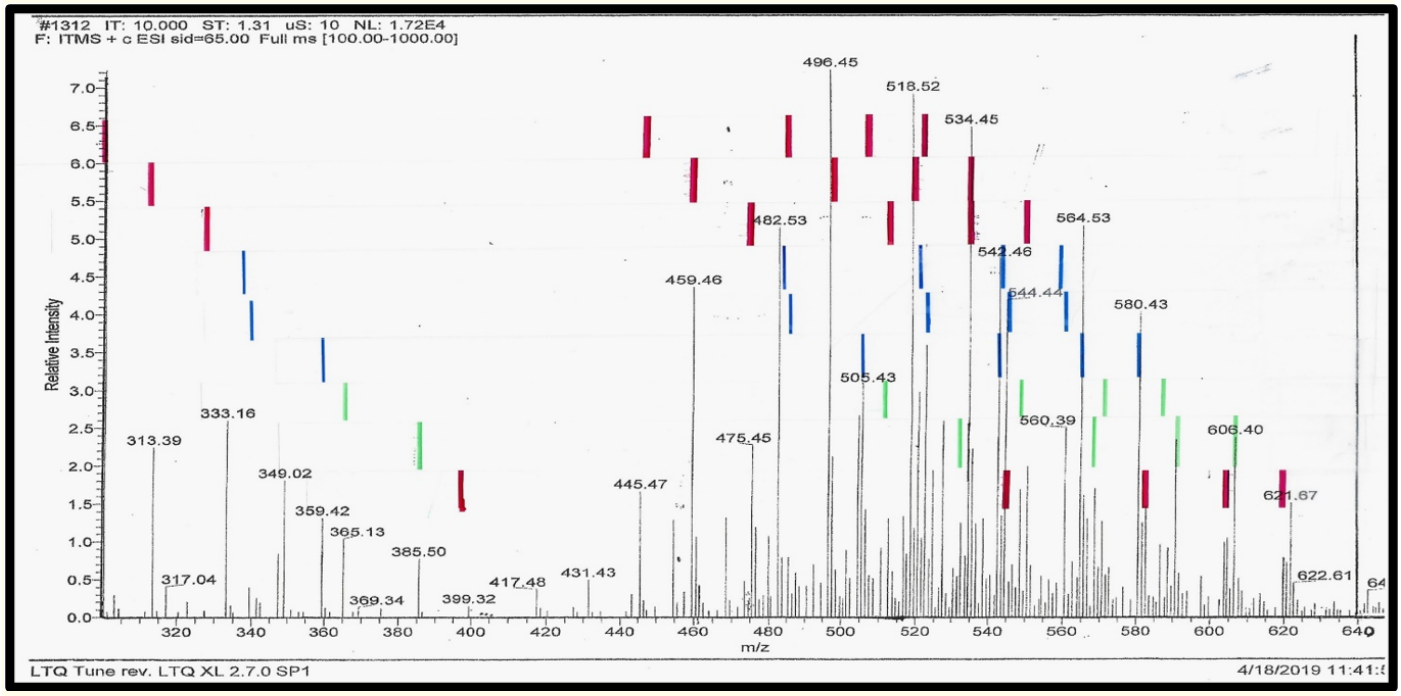

Figure 2: *Mass spectrum from an extract prepared from Pacific oysters (Crassostrea gigas).*
The spectrum is overlaid with the projected pattern associated with each S ion, even though not all of the S ions were detectable in this spectrum. Table 2 lists the ions expected for each S ion. Fig. 3 is a bar graph showing the relative intensities of the ions associated with each S. No similar pattern was associated with the ions at 317, 333, 349 or 369 Da. Their origin is unclear.
Group 1: 21 carbon steroids; red; 299. 313, 329.
Group 2: 23 carbon steroids; blue; 337. 339, 359.
Group 3: 24 carbon steroids; green; 365, 385.
Group 4: 25 carbon steroids; red; 399.

Table 2 lists the S ions and the expected phosphate-containing fragments. Figure 4 plots the m/z ions for each S fragment color coded for the number of carbon atoms in S: red ions have 21 carbon atoms; green ions have 23 carbon atoms; green ions have 24 carbon atoms; blue ions have 24 carbon atoms; and red ions 25 carbon atoms. Using the same color codes, A, B, C and D fragments for most of the S values at 299, 313, 359, 365, 385 and 399 were present in the mass spectrum. More than 30 of the ions can be attributed to S phosphocholine esters or fragments. Two peaks, characteristic of S = 339 and S = 341, were observed, even though the parent S ions had very low RI or were undetectable. Ions corresponding to 333 Da and 349 Da could not be matched with phosphocholine parents. Their origin is unclear.

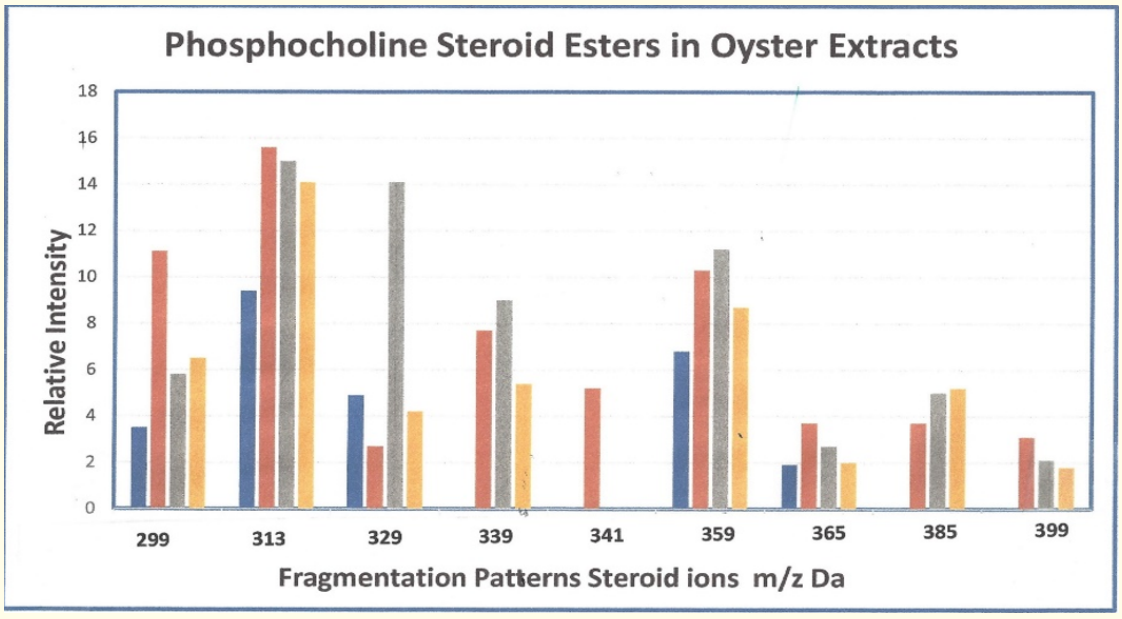

Figure 3: *Phosphocholine ions detected in Oyster extracts.*
Each cluster shows the relative intensity of A, B, C, and D fragments for each phospho-steroid fragment.
The B fragment of C341 at m/z=524 Da may not be related to C341 as no related fragment was identified.
The D fragment of C313 and the C fragment of C329 have the same mass, m/z = 534 Da. This probably accounts for the high intensity of C fragment of C329 when compared to the other fragments of C329.

S				
m/z				
Da	A	B	C	D
	S+146	S+183	S+205	S+221
299	445	482	504	520
313	459	496	518	534
329	475	512	534	550
339	485	522	544	560
341	487	524	546	562
359	505	542	564	580
365	511	548	570	586
385	531	568	590	606
399	545	582	604	620

Table 2: *List of S compounds in oyster extracts.*

This table list the S compounds and A, B, C, and D fragments as detected by mass spectrometry. Each line has the mass of the of the steroid fragment and its expected phosphate containing fragments as detected by mass spectroscopy. Column B list the masses of the H+ ion. Column C lists the masses of the Na^+ ion. Column D lists the masses of K^+ ion. Column A lists the masses of the Na+ ion after loss of the trimethylamine from the choline fragment. See figure 3 for the relative intensity of the ions derived from each S.

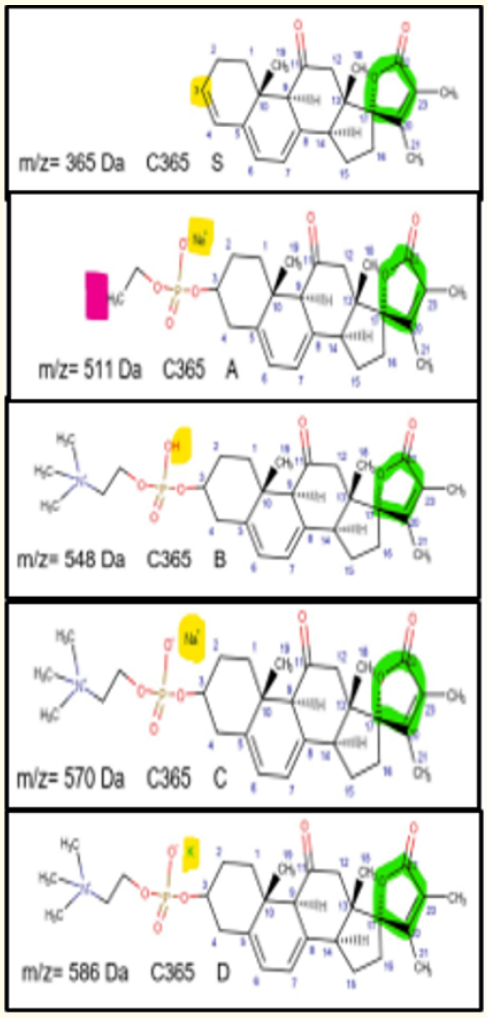

Figure 4: *Proposed structure for C365 and its phosphocholine ions.*
From Top to Bottom: S, A, B, C, D.
In each panel, the spiral lactone ring is highlighted in green and the cation is highlighted in yellow.
The red highlight in panel A identifies the loss of trimethylamine (59 Da) from C.
Features of note: [1] C365 has 24 carbon atoms. [2] There are ions present in the mass spectrum corresponding to each proposed structure. Occam's razor suggests the stereochemistry will be the same for all 5 compounds. [3] The feature at carbon 11 is a ketone, rather than an alcohol. [4] There were no compounds with either of the ring B double bonds reduced, yet oysters make cholesterol. This suggests that the 7-dehydrocholestrol reductase in oysters does not reduce the phosphocholine steroid esters.

Overall, this data is not intended to quantitate the individual compounds but only to confirm their existence. Although we have proposed structures for each of the steroid fragment ions, whether or not the proposed structures are absolutely correct in their stereochemistry, doesn't change the fact that they represent a novel type of phosphocholine steroid. Thus, phosphocholine steroid esters are present in non-vertebrates and they should not be considered as solely a hormone of warm-blooded vertebrates.

Discussion

Without the knowledge of the characteristic fragmentation patterns of relatively pure steroid phosphoesters from mammals [1], it would not have been possible to recognize the novel compounds present in the oyster extracts. We had also synthesized a model steroid phosphocholine ester and confirmed that it had both a similar ^{31}P NMR spectrum and a similar fragmentation pattern. These observations were the basis for this investigation. As shown in table 2, there were 9 phosphocholine compounds in the extract with m/z ranging from C299 to C399. Table 3 applied the trial and error method to determine the likely chemical composition of C365, C385, and C399.

Table 3, Panel A, shows a trial and error analysis of all possible compositions for C365. The composition with a molecular mass of 382 Da fits with $C_{24}H_{30}O_4$. The figure legend details how the trial and error result was obtained. Note that carbon-17 is part of two rings - Ring D and Ring E - and thus, is a spiral steroid. In table 2 and figure 3, C365 was matched with its phosphocholine parent ions. This confirmed

Panel A Analysis of m/z = 382 Da-C 365							
Line	**Carbons atoms**	**Oxygens atoms**	**C&0 Da**	**Desired Da**	**H Req**	**H Max**	**Delta**
1	23	4	340	382	42	48	3
2	23	5	356	382	26	48	11
3	24	3	336	382	46	50	2
4	24	4	352	382	30	50	10
5	25	3	348	382	34	52	9
6	25	4	364	382	18	52	17
7	26	2	344	382	38	54	8
8	26	3	360	382	22	54	16
Panel B Analysis of m/z = 400 Da-C 385							
Line	**Carbons atoms**	**Oxygens atoms**	**C&0 Da**	**Desired Da**	**H Req**	**H Max**	**Delta**
1	23	5	356	400	44	48	2
2	23	6	372	400	28	48	10
3	24	5	368	400	32	50	9
4	24	6	384	400	16	50	17
5	25	4	364	400	36	52	8
6	25	5	380	400	20	52	16
7	26	3	360	400	40	54	7
8	26	4	376	400	24	54	15

Panel C Analysis of m/z = 382 Da-C 365							
Line	**Carbons atoms**	**Oxygens atoms**	**C&0 Da**	**Desired Da**	**H Req**	**H Max**	**Delta**
1	24	5	368	414	46	50	2
2	24	6	384	414	30	50	10
3	25	5	380	414	34	52	9
4	25	6	396	414	18	52	17
5	26	4	376	414	38	54	8
6	26	5	392	414	22	54	16
7	27	3	372	414	42	54	6
8	27	4	388	414	26	54	14

Table 3: *Trial and error analysis of C365, C389, and C399.*

# of Carbon: Proposed number of carbon atoms (C) in molecule.

# of Oxygen: Proposed number of oxygen atoms (O) in molecule.

Da - C&O: Contribution to # of Da for the specified number of C and O atoms.

Desired Da: Number of Da in the molecule as indicated by the MS data.

Hreq: Number of H required to complete the molecule after the C and O contribution.

Hmax: Maximum number of H possible based on the number of C atoms (2N+2).

Delta: For each 2 H atoms in the difference between Hreq and Hmax there must be one double bond or ring (Delta).

The line in red on each panel shows the composition most consistent with the steroid fragment identified by mass spectroscopy.

its status as a phosphocholine spiral steroid with 24 carbon atoms.

Table 3, Panel B shows a trial and error analysis for *C385. $C_{24}H_{32}O_5$. The extra oxygen suggests that the E-Ring has not been formed. If the compound was a carboxylic acid, it would be an anion as drawn. In order to be detected as a positive ion, it would have to add two hydrogen atoms as it was analyzed in the mass spectrometer. Thus, the *C385 cation is generated by a formula with m/z = 400 Da +2 hydrogen atoms less 17 Da from loss of the phosphocholine fragment attached to carbon-3.

Table 3 Panel C shows a similar analysis for C399. $C_{25}H_{34}O_5$. This composition produces a molecule with a mass of 414 Da and would produce a fragment at 399 Da but only if the fragment was a carboxylic acid. In summary, *C399 seems to be a carboxylic acid with 25 carbon atoms but the corresponding spiral steroid was not detected in the extract.

Biosynthesis

When only phosphoesters with 21 and 23 carbon atoms were known, we had proposed that the two extra carbon atoms were added by a malonyl-Co-Enzyme A condensation [1]. Now that we know about the compounds with 24 and 25 carbon atoms, that theory can no longer explain the diversity of phosphocholine steroid esters and their precursors. The new theory is that the extra carbons are added from acyl-CoEnzymeA derivatives. Table 4 lists the common acyl derivatives and the corresponding products. Figure 5 shows the proposed structures for the seven phosphocholine steroid esters that we identified. Six of the 7 (maybe all 7) have the 5-7 diene structure similar to that of 7-dehydrocholesterol. Apparently, the 5-dehydro-reductase and the 7-dehydro-reductase enzymes that reduce phosphoesters are only present in minimal amounts in oysters, if at all. Note that oysters do make cholesterol, indicating that an enzyme that reduces

Acyl derivative	R	# of C	Carboxyl	Spiral lactone	Acceptor
Acetyl-CoA	# H	23	C359	C339	C313
Propyl-CoA	# CH_3	24	C385@	C365@	C329@
Acetoacetyl-CoA	# $COCH_3$	25	C399	(C379)	C313

Table 4: *CoEnzyme A condensation with C313 or C329.*

Notes: Column R lists the substituents on Carbon 23.

[#] These three are the most common CoA derivatives.

@ To account for the extra 16 Da on these three compounds, the steroid must have an extra hydroxy.

group. Based on known enzymatic processes, these could be 11β-hydroxylated or 11-keto steroids. 11-hydroxylation might occur before or after the condensation with the acyl-CoA.

7-dehyrocholesterol is present but the phosphocholine group may prevent binding to the active site.

Endocrinology

The original discovery of the spiral steroids was based on [a] the presence of an unknown digoxin-like material in human breast cyst fluids with high K^+ levels (4) and [b] its absence in patients with 7-dehydro-reductase deficiency (Smith-Lemli-Opitz syndrome) [5,6]. On the basis of its structural similarity to both spironolactone and ouabain, we proposed it was the endogenous cardiotonic steroid and that it functioned as a potassium sparing hormone.

Several laboratories have suggested that the human digoxin-like material is actually ouabain [7] or a marinobufagenin-like material [8]. However, a biosynthetic path for neither of these compounds has been proposed [9]. Neither compound can be detected in serum samples by any method other than by immunoreactivity with antibodies made to the postulated compound. In contrast, in papers published in Steroids, we have isolated mg quantities of spiral steroids [1]. ^{31}P-NMR data confirmed the presence of a phosphate in the molecule. Fragmentation indicated the molecule was a phosphocholine. LC-MS on small aliquots of serum (0.2 ml) demonstrated peaks consistent with the pure material [10]. The other groups have no similar data confirming the existence of either ouabain or marinobufagenin in mammalian or avian serum or tissue extracts. Despite these facts, some investigators maintain their belief. Nichols suggests that endogenous ouabain is a fantasy [11,12].

Biology

Although the genome for oysters has been sequenced, it is unlikely that oyster physiology is going to be thoroughly investigated any time soon [13]. I chose to investigate the phosphocholine steroid esters in oysters primarily to determine if these compounds existed in invertebrates or if they were limited to vertebrates. An extract of the whole oyster was sure to include the site of synthesis, metabolism and/or storage. As oysters live in a salt-water bath (sea water), they must concentrate potassium against a strong gradient, just like the human fetus [14]. In fact, C339 was present, but there was at least one other spiral lactone, C365, and several steroid carboxylic acids *C359, *C385 and *C399 that had not previously been detected in mammals.

Speculation

Oysters are filter feeders that live in salt water. In fact, prevention of salt accumulation is probably more of an issue than is salt wasting. In humans, aldosterone functions by stimulating production of the epithelial sodium channels via the mineralocorticoid receptor. An

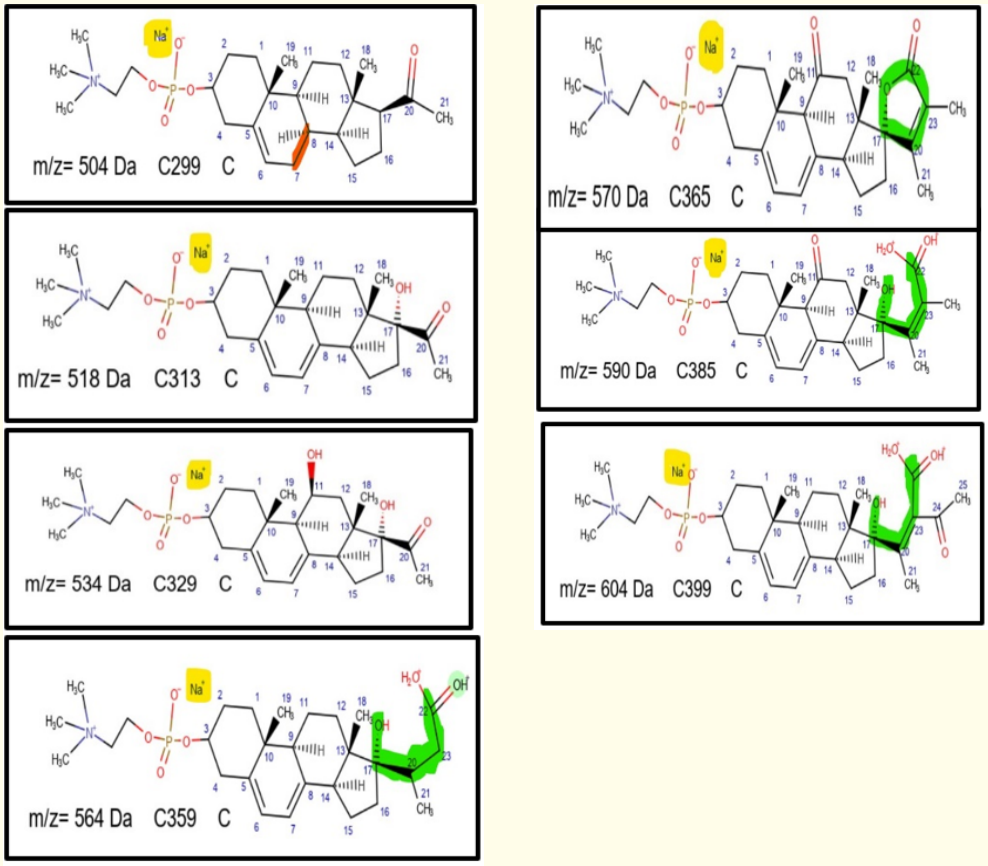

Figure 4: *Proposed structures for the phosphocholine esters in oyster extracts.*
From top to bottom: These are the Na+ ions of the seven steroid phosphoesters for which there were at least three (of the four) unambiguous, positive ions. In each panel, the Na+ ion has been highlighted in yellow and the ions that form the E-ring are highlighted in green. The red highlight marks the Δ7-8 double bond in 7-dehydrocholesterol or 7-dehydropregnenolone.

inborn error, syndrome of Apparent Mineralocorticoid Excess (AME), is caused by an inability to convert 11β-hydroxy steroids to 11-keto steroids. This prevents synthesis of epithelial sodium channels [15]. If the proposed structure of C365 is correct, it is an 11-keto steroid and might prevent synthesis of epithelial sodium channels. In summary, C365 could be the hormone necessary to prevent sodium accumulation in oysters, despite their filter feeding in salt water.

Summary

Spiral steroid phosphocholines are widely distributed in the animal kingdom and are not limited to warm-blooded vertebrates. As there are spiral steroids with different carbon backbones, the compounds may have different functions, just like the classical steroid hor-

mones with 18, 19, or 21 carbon atoms all have different functions.

Highlights

- Oysters make three classes of spiral steroids distinguished by the number of carbon atoms in the steroid.
- Each of the three classes, characterized by 23, 24, or 25 carbon atoms, had a carboxylic form but we only detected spiral steroid phosphocholine esters with 23 and 24 carbon atoms.
- The extra carbons seem to be added by condensation of the corresponding acyl-CoEnzyme A with a 21-carbon phosphoester precursor.
- Spiral steroid phosphoesters are more widespread than just mammals and birds.

Acknowledgements

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Nomenclature

Individual phosphocholine steroid esters are named by the mass of the S fragment with the format Cxxx, where xxx is the actual m/z detected by the mass spectrometer. *Cxxx indicates the mass is most consistent with a carboxylic acid with the extra carbon atoms not completing the spiral lactone E-ring.

Declaration of Competing Interests

None.

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