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LIPID ANALYSIS OF VERTEBRATE COPROLITES

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Abstract—Lipid analysis is a relatively new approach to obtaining paleobiological and paleoecological information from coprolites. Lipids in feces are derived from multiple sources, including diet, digestive processes and digestive tract micro-organisms. Feces from herbivorous animals contain a much wider range of lipids than those from carnivores due to the greater diversity of lipids in dietary plants, compared to prey animals, and the more complex digestive systems of herbivores. Depending on their structure and their preservation in fossilized material, fecal lipids can provide general or very specific biological and ecological information. Reports concerning the lipid analysis of coprolites have been scarce until recently, but this approach has the potential to reveal unique information about ancient animals and environments and should therefore be considered as a valuable tool for analysis given suitable specimens.

INTRODUCTION

Since organic geochemistry first became established, one of its main aims has been to use organic molecules, preserved in ancient materials, to obtain insights into aspects of life in the geological past. Initial research efforts focussed on petroleum and ancient marine/lacustrine sediments as repositories of "fossil molecules," but as the discipline has developed, analysis of other materials, including fossils, has become possible. The organic geochemistry and, in particular, the lipid content of coprolites has been the subject of relatively few studies to date. However, this approach to coprolite analysis offers great potential to reveal aspects of the biology and ecology of extinct animals.

Herbivore coprolites are generally scarcer in the fossil record than those of carnivores, because the phosphate content of the latter from the soft tissue and bones of prey animals predisposes them to mineralization (Thulborn, 1991). However, herbivore coprolites have the potential to contain a much more diverse array of lipids, due to greater chemical variation in their diet and because of their more complex digestive systems and processes, and are therefore the main focus of this short review.

LIPIDS AND BIOMARKERS

Lipids are broadly defined as hydrophobic small molecules. Within this very general grouping, there is a great diversity of chemical structure, ranging from simple, straight carbon chains (e.g., *n*-alkanes) to complex molecules containing multiple ring structures and functional groups (e.g., conjugated bile acids). Lipids are commonly, but not exclusively, derived from the cell membranes of plants, animals and microorganisms. Although the terms lipid and fat are sometimes used as synonyms, in chemical terms fats (triglycerides) are a subgroup of lipids. Figure 1 shows some examples of lipids commonly observed to occur in feces. The structures of all lipids mentioned in the text are listed in Appendix 1.

In the context of organic geochemistry, biomarkers are molecules of biological origin that can be unequivocally linked to a source or process on the basis of chemical structure and/or stable isotope composition. Many biomarkers are lipids and many lipids are biomarkers, but there are exceptions in both cases and the terms should not be used interchangeably. This use of the term "biomarker" in a geochemical context should also not be confused with its use in medicinal studies where it is a moniker applied to compounds that provide an indication of a physiological condition (Atkinson et al., 2001). Molecules in the cells of living organisms ("biomolecules") may undergo structural modifications, e.g., loss of functional groups, during the processes that lead to preservation in the geological record (where they can be referred to as "geomolecules"). However, if the carbon skeleton of the molecule contains sufficient structural and/or stable isotopic information to link it to the original biomolecule, then it can still be used as a biomarker.

Sources of Lipids in Feces

The lipid content of feces is a product of both the lipids introduced into the digestive tract and the physical, chemical and biological processes that act to modify those molecules before excretion (Fig. 1). Sources of fecal lipids include diet, the organism producing the feces and digestive tract micro-organisms (Leeming et al., 1996).

Diet

Diet is a major source of fecal lipids. Dietary lipids from carnivores mainly consist of cholesterol (I) and its derivatives, from the cells of prey animals. Feces from herbivores, the main subject of this review, contain lipids from dietary plants, which can be highly diverse (e.g., Jansen et al., 2006).

A variety of compounds, or suites of compounds, that are commonly present in feces indicate a general herbivorous lifestyle. For example, phytosterols such as campesterol (II), sitosterol (III) and stigmasterol (IV), which are derived from plant cell membranes and are analogous to cholesterol (I) in animal tissues, are ubiquitous in herbivore feces. Indeed, these compounds and their saturated analogs 5ß-campestanol (V) and 5ß-stigmastanol (VI), together with 5ß-cholestanol (coprostanol) (VII) derived from cholesterol have been used to identify fecal pollution from herbivores in wastewaters (Leeming, 1996; Bull et al., 1998, 2002). Other general indicators of an herbivorous diet include a series of long-chain *n*-alkanes with an odd-over-even carbon number distribution, e.g., C_{25}, C_{27}, C_{29}, C_{31} (VIII), and a corresponding series of *n*-alkanols with an even-over-odd carbon number preference, e.g., C_{26}, C_{28}, C_{30}, C_{32} (IX). These compounds are constituents of epicuticular leaf waxes and the characteristic carbon number distribution is a direct consequence of their biosynthetic origin (Eglinton and Hamilton, 1967). Sometimes the maxima of these series can be broadly indicative of a particular group of plants, e.g. the C_{26} *n*-alkanol for grass (van Bergen et al., 1997).

Other groups of compounds are restricted to particular taxa of plants and therefore provide more detailed dietary information. For example, pentacyclic triterpenoids e.g., ß-amyrin, lupeol and their derivative oleanane (X) are indicative of angiosperms (Moldowan et al., 1994) and have been recovered from coprolites (e.g., Van Geel et al., 2008). Tricyclic diterpanes e.g., ferruginol (XI), are derived from gymnosperms including conifers (e.g., Otto et al., 2001).

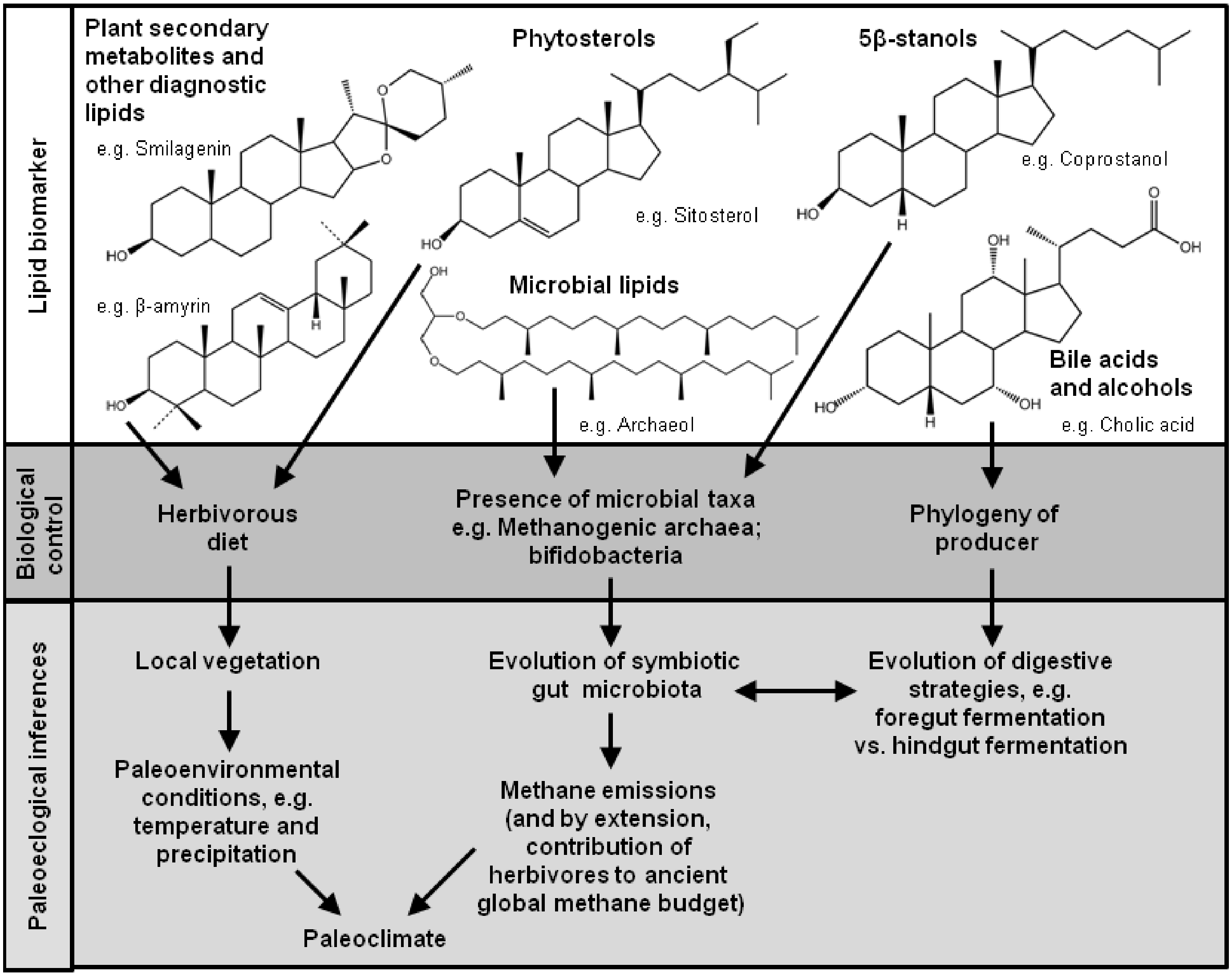

FIGURE 1. Relationship between lipid biomarkers in feces, biological controls and palaeoecological inferences.

Some individual lipids are restricted to an even narrower range of plants. For example, epismilagenin (XII), a spiroketal sapogenin, was found in an 11,000 year old ground sloth coprolite (Fig. 2). Spiroketal sapogenins are secondary metabolites typically produced by monocot plant families (Dewick, 2009) and in this instance the epismilagenin was interpreted to have derived from digestive processing of *Yucca* or *Agave* leaves (Gill et al., 2009).

Producer

A significant component of fecal lipids comes from the actual animal that produces the feces. For example, even when cholesterol is not consumed in the diet, cholesterol (I) and its saturated analogs, e.g., coprostanol (VII), are common components of the fecal lipid signature, interpreted to derive from endogenous sources, such as cells of the digestive tract lining that are sloughed off during passage of food through the gut (Ferezou et al., 1978).

Bile acids are produced by animals to assist with the breakdown of fats in the diet and to regulate cholesterol levels (Hofmann, 1999). Primary bile acids, e.g. chenodeoxycholic (XIII) and cholic (XIV) acids, are formed in the liver and are modified in the digestive tract to form secondary bile acids, e.g., lithocholic (XV) and deoxycholic (XVI) acids. Certain taxa produce characteristic suites of bile acids (e.g., Hagey et al., 2010), which have been used in conjunction with other lipids to identify sources of fecal pollution in both modern and ancient settings (Elhmmali et al., 1997; Bull et al., 1998, 1999a, 2002). Elephants, hyrax and manatees are unique among mammals in that they produce only bile alcohols and do not produce any bile acids (Hagey et al., 2010). This is a feature that appears to have been shared by mammoths as well, since bile acids have not been detected in well preserved coprolites and digestive tract contents from mammoths (Van Geel et al., 2008, 2011). Thus, bile acids in coprolites can provide some insights into the phylogenetic affinities of the producer.

Digestive Tract Microbes

Digestive tract microbes contribute to the fecal lipid signature both directly, in lipids derived from their cell membranes, and indirectly by modifying lipids derived from the diet and the producer. For example, cholesterol and the higher plant analogs, campesterol and sitosterol, are hydrogenated in the digestive tract by bifidobacteria to give saturated analogs with a specific stereochemical configuration, 5ß-stanols (Murtaugh

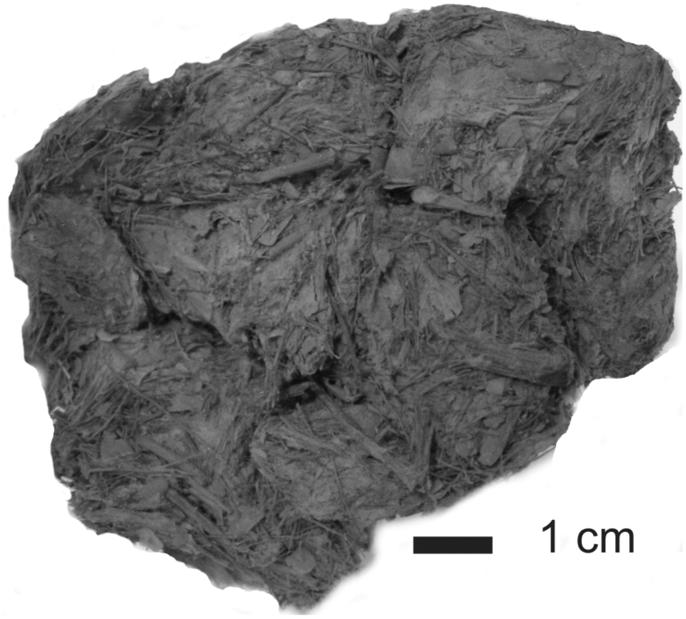

FIGURE 2. Desiccated coprolite BRSUG 19569-3, Gypsum Cave, Nevada, attributed to *Nothrotheriops shastensis*.

and Bunch, 1967). Similarly, primary bile acids are modified by digestive tract bacteria to produce secondary bile acids (Aries and Hill, 1970).

Although generally in lower relative abundance than the other classes of compound, lipids derived directly from micro-organisms in the digestive tract can provide some of the most useful information available from feces. Since no mammals have been found to produce the enzymes necessary to break down plant structural polysaccharides, such as cellulose, herbivorous mammals are obliged to live in symbiosis with micro-organisms that can perform this function. This has led to various modifications of the digestive tract in different groups of herbivores, e.g., the evolution of foregut fermentation in the artiodactyls and the enlargement of the caecum or colon in the perissodactyls (Janis, 1976). Some of these differences are reflected in the fecal lipid signature. For example, the compound archaeol (XVII) was detected in the feces of foregut fermenting mammalian herbivores, but not in hindgut fermenters, suggesting that archaeol in coprolites might represent a biomarker for foregut fermentation (Gill et al., 2010). Archaeol is a dialkyl glycerol ether (DAGE), a ubiquitous component of archaeal cell membranes, but in feces is interpreted to be derived specifically from methanogenic archaea. Recently, a correlation was established between methane emissions and faecal archaeol concentration in modern cattle (Gill et al., 2011), raising the possibility that archaeol preserved in coprolites could be used to calculate methane emissions from extinct herbivores.

Other microbial lipids found in feces include fatty acids, which may be characteristic of particular microbial taxa. For example, straight chain and branched C_{15} (XVIII, XIX) and C_{17} fatty acids, some of which have been found in mammoth intestinal contents (Van Geel et al., 2008), occur in cellulose-degrading and starch-degrading bacteria (Vlaeminck et al., 2004).

METHODOLOGY

Lipids can be extracted from modern feces, desiccated dung and lithified coprolites. Although the details of the extraction methods vary according to the material analyzed, the general approach remains the same. On the principle that "like dissolves like," organic solvents such as dichloromethane, chloroform and methanol are used to extract lipids from dried material, to give a total lipid extract (TLE). This is then separated into fractions according to the chemical properties of the compounds, i.e., which functional groups they possess, using column chromatography. This involves passing the TLE through a glass column packed with a stationary phase, usually alumina or silica, and eluting the desired fractions using a sequence of solvents of increasing polarity. In some cases, it may be desirable to separate fractions still further, for example an alcohol fraction may be separated into straight-chain and cyclic alcohols by urea adduction. Depending on the method of analysis, fractions may be derivatized to replace reactive functional groups such as hydroxyl moieties with less reactive groups such as trimethylsilyl moieties. Standard methods exist for analysis of specific groups of fecal biomarkers such as sterols (Bull et al., 1999b) and bile acids (Elhmmali et al., 1997).

Analytical methods commonly used for fecal lipids include gas chromatography (GC), gas chromatography-mass spectrometry (GC/MS) and gas chromatography-isotope ratio mass spectrometry (GC/IRMS). For samples in which the compounds of interest are in low abundance, specific techniques such as selected ion monitoring gas chromatography–mass spectrometry (SIM-GC/MS) or gas chromatography-tandem mass spectrometry (GC/MS) may be employed in order to lower the limit of quantification (Evershed and Bethell, 1996).

HISTORY OF LIPID BIOMARKER ANALYSIS OF COPROLITES

The term coprolite was coined by William Buckland (Buckland, 1829) and since then coprolites have been widely studied. However, until recently, relatively few studies have included lipid analysis of coprolites.

In one of the earliest applications of analytical chemistry to the study of coprolites, Lin et al. (1978), analyzed lipids from human coprolites originating between 50 AD and 100 BC. The predominant compounds recovered were steroids of animal and plant origin (i.e., cholesterol and phytosterols and corresponding 5ß-stanols) and bile acids, including lithocholic acid and deoxycholic acid. These compounds were found in the same relative abundance in the coprolites as in modern human feces, but the absolute abundance of the lipids in the coprolites was considerably lower, with mean values for the total steroid content of the coprolites approximately one third of those for modern feces (~9000 μ/g versus ~28000 μ/g).

One of the earliest published references to lipid analysis of coprolites of geological age is that of Chin and Brassell (1993) where the biomarker content of Mesozoic coprolites from a carnivore and several herbivores is described. Chin (1996) provided details about the lipid content of eight Mesozoic coprolites, including five herbivore coprolites. The herbivore coprolites contained n-alkanes and steranes indicative of an herbivorous diet (C_{27}, C_{29} and C_{31} *n*-alkanes, C_{29} sterane) and some contained oleanane and derivatives (from angiosperms) and tricyclic diterpanes (from gymnosperms), consistent with the coniferous wood fragments preserved in the sample.

Bile acids were analysed by nuclear magnetic resonance (NMR) from coprolites from five extinct herbivore taxa (*Mammuthus* sp., *Oreamos harringtoni*, *Euceratherium collinum*, *Symbos* sp. and *Nothrotheriops shastensis*), as well as feces from 16 modern species from the Colorado Plateau, USA, by De Ropp et al. (1998). However, the spectra from different taxa showed considerable similarities and could not be used to identify the producer, highlighting the limitations of this analytical approach.

Hollocher et al. (2001) analyzed herbivorous dinosaur coprolites from the Cretaceous Two Medicine Formation by pyrolysis-gas chromatography-mass spectrometry (py-GC-MS). Pyrolysis thermally decomposes bulk organic matter (i.e., lipids plus organic macromolecules), which is a different approach to the studies mentioned so far, in which lipids have been extracted and separated from the bulk organic matter before analysis. Hollocher et al. (2001) interpreted the pyrolysis products to be mainly of bacterial origin, with some contribution from higher plants, whereas lipids extracted from the same coprolite (Chin et al.,

1993) were almost exclusively attributed to higher plants. Lipids comprise only a small fraction of total organic matter in coprolites so their isolation and analysis by GC-MS may provide more detailed insights than analysing bulk organic matter by py-GC-MS, when the lipid signal may be swamped by the presence of other organic components.

Lipid analysis of lower intestine contents from a 22,500 year old woolly mammoth (*Mammuthus primigenius*) preserved in permafrost in Russia was carried out as part of a multidisciplinary study (van Geel et al., 2008). The lipid components were almost exclusively of plant origin and included odd-numbered *n*-alkanes, even numbered *n*-alkanols maximising at C_{26} (indicating a significant contribution of grass to the diet), phytosterols and corresponding 5ß-stanols and pentacyclic triterpenoids. The intestine contents were analyzed for bile acids, but none were detected. Non-lipid evidence suggested that the mammoth had ingested dung and the absence of bile acids was interpreted to indicate that the dung ingested was from a mammoth.

A similar multidisciplinary study was conducted on 12,500 year old mammoth coprolite from Alaska (van Geel et al., 2011). The lipid content of the coprolite was very similar to that from the Russian mammoth intestine contents (van Geel et al., 2008) and was dominated by plant-derived compounds, again with a complete absence of bile acids. One difference was the presence of unsaturated C_{18} fatty acids and C_{27}-$_{29}$ Δ^{22}-5ß-stanols in the Alaskan coprolite, also of plant origin and indicative of the high level of preservation exhibited by the material.

The dominant lipid in an 11,000 year old sloth coprolite was found to be epismilagenin (XII), a spiroketal sapogenin (Gill et al., 2009). This compound was interpreted to have derived from digestive processing of a plant containing the precursor molecules smilagenin or diosgenin (Miles et al., 1992). The list of plants containing one or both of these molecules is restricted to about 20 taxa and when palaeoenvironmental considerations were taken into account, the most likely sources were found to be *Yucca* spp. and *Agave* spp.

Hagey et al. (2010) carried out an extensive survey of bile acids and bile alcohols from 219 reptile species and 326 mammalian species and also analyzed the bile content of an 8,000 year old human coprolite from the Danger Cave archaeological site, Utah, USA, and a coprolite attributed to the Shasta ground sloth (*Nothrotheriops shastensis*) from Rampart Cave, Arizona, USA. The human coprolite contained a suite of primary and secondary bile acids, including cholic acid, lithocholic acid and deoxycholic acid, which is similar to that in modern human fecal contents. The sloth coprolite, which was between 11,000 and 35,000 years old, contained a series of glycine-conjugated mono-, di-, tri- and tetra-hydroxylated C_{24} bile acids, consistent with the bile acid profiles of modern tree-sloth species. These results demonstrate that bile acids, as well as sterols and other lipid biomarkers, can be preserved in coprolites and may be of use for paleontological studies.

PROSPECTS AND CONCLUSIONS

Although lipid analysis is still a relatively novel method of investigating coprolites, and has inherent limitations, it has the potential to provide unique insights into the diet, digestive processes and digestive tract microbiota of extinct animals. The major limitation is the amount of original organic material available for analysis in ancient feces. Concentrations of specific fecal lipids can range from close to modern values to more than an order of magnitude lower in desiccated archaeological and geological samples (e.g., Lin et al., 1978; Van Geel et al., 2008; Gill et al., 2010). In lithified coprolites total organic carbon content (of which lipids may be a relatively minor component) is typically extremely low, e.g., 0.85% for a dinosaur coprolite versus 30.5 % for modern reptile feces (Chin and Brassell, 1993). This means that large volumes of material, up to 40 g (Chin, 1996), are required for lipid analysis of lithified coprolites, whereas sample sizes for modern and desiccated fecal material can be much smaller (0.5-2g). Since lipid analysis is a destructive technique, requiring that the specimen be powdered before extraction, it is unsuitable for small, rare or precious specimens. For these reasons, lipid analysis is perhaps most suitable for analysis of desiccated fossil or sub-fossil feces from sites with abundant material, e.g., the abundant herbivore coprolite deposits preserved in the caves in the USA (Mead and Agenbroad, 1992). Ideally, lipid analysis should be carried out as part of a larger, multidisciplinary study of coprolites, so that inferences from palaeobotany, palynology, DNA analysis and other approaches can be validated by lipids results, and vice versa.

In spite of the limitations described the unique insights lipid analysis can provide into paleobiology and paleoecology mean that for suitable specimens it should be considered a valuable addition to existing methods of investigating coprolites.

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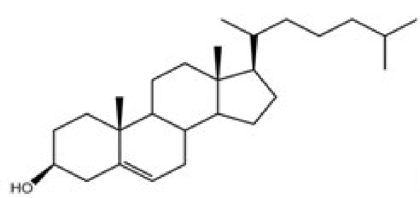
APPENDIX 1 - STRUCTURES CITED IN THE TEXT.

I



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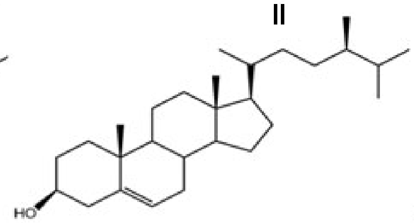

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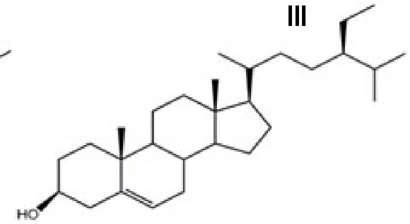

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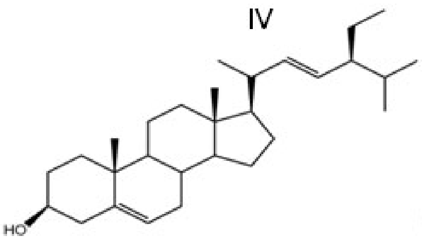

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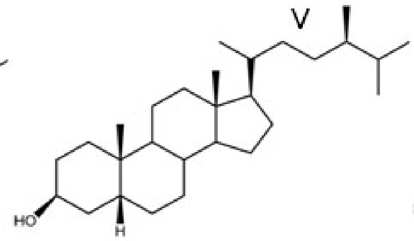
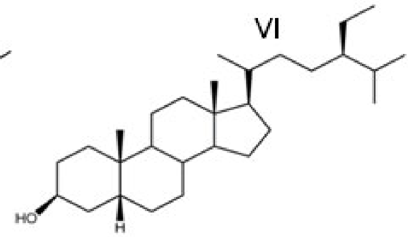


VI



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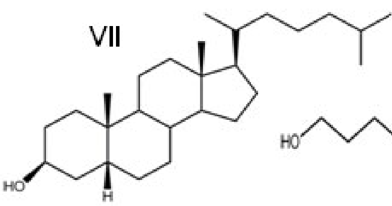

VIII

IX

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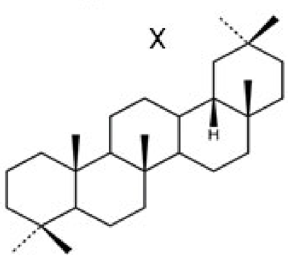

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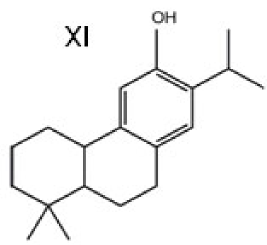

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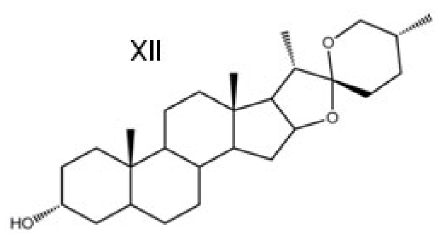

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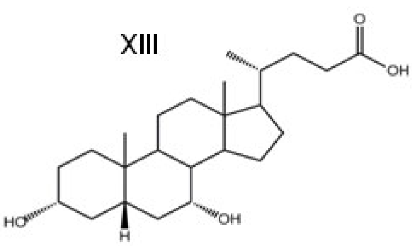

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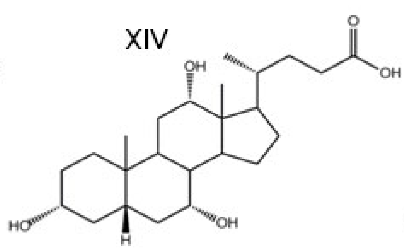

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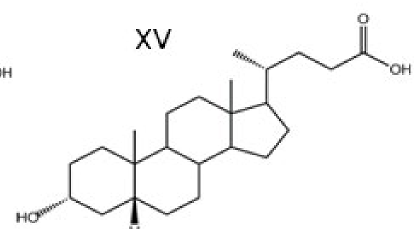

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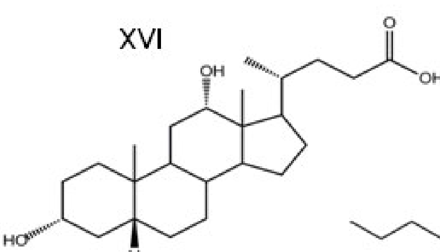

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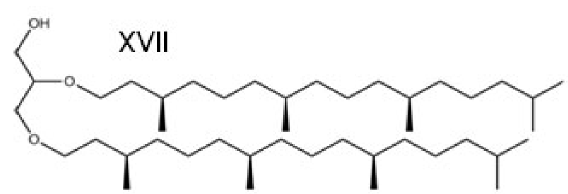

XVIII

XIX