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(54) **Title:** NEW BIS ESTERS OF IVY SAPOGENINS FOR RUMINANTS

(57) **Abstract:** The present invention relates to synthetic bis esters of hederagenin, and their use in ruminants to improve ruminant growth performance, reduce rumen methane emission, reduce urine ammonia excretion, and/or to reduce rumen acetate to propionate ratio. Moreover, it also relates to a feed composition for ruminants comprising bis esters of hederagenin, and to novel bis esters of hederagenin.



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## **NEW BIS ESTERS OF IVY SAPOGENINS FOR RUMINANTS**

### **Technical Field**

5 The present invention relates to the field of synthetic bis esters of ivy sapogenins, and their use in ruminants to improve ruminant growth performance, reduce rumen methane emission, reduce urine ammonia excretion, and/or to reduce rumen acetate to propionate ratio. More particularly, it relates to bis esters of hederagenin and their use. Moreover, it also relates to a feed composition for ruminants comprising bis esters of hederagenin, and  
10 to novel bis esters of hederagenin.

### **Background of the invention**

Saponins are secondary compounds found in many plants. They form a stable foam in  
15 aqueous solutions such as soap, hence the name "saponin". Chemically, saponins as a group include compounds that are glycosylated steroids, triterpenoids, and steroid alkaloids.

There have been several reviews in recent years about the implications or applications of  
20 saponins in animal systems or production. As an example, yucca and Quillaja saponins have been shown, under specific conditions, to be beneficial for rumen fermentation. Nevertheless, their effect is generally not sustained over a long period of time. Ivy saponins (hederagenin) have so far not been tested in ruminants.

25 Protozoa form integral part of the microbial flora of ruminants together with bacteria, fungi, and archaea. Protozoa are well known predators of bacteria causing a rapid turnover of protein within the rumen and decreasing the supply of bacterial protein to the ruminant host. In addition, protozoa are indirectly involved in methane production resulting from ruminant digestion, as methanogenic archaea live in symbiotic association with protozoa.  
30 Thus elimination of ciliate protozoa from the rumen would lead to an increased production efficiency and sustainability of domestically supplied ruminant meat and milk for food, whilst reducing greenhouse gas emissions from the supply chain.

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Saponins are well known strong antiprotozoal agents that have been described to strongly inhibit ciliate protozoa in the rumen without affecting the bacterial microflora (Hart, *et al.*, Plant extracts to manipulate rumen fermentation. Animal Feed Science and Technology. 147 (2008) 8–35). However, saponins have never been commercially developed as  
5 successful product to reduce ruminal protozoa populations thereby leading to improved ruminant productivity. Indeed, it is believed that the very complex and diverse rumen microflora rapidly adapts and develops so as to provide enzymes able to cleave the glycosidic bond of the saponin leading to the sapogenin (aglycone form of the saponin) which is not active in inhibiting protozoa. As a result, when feeding ruminants with  
10 saponins, the effect of saponins does not persist more than about 1 week (Ivan et al., “Effect of the dietary *Enterolobium cyclocarpum* foliage on the population dynamics of rumen ciliate protozoa in sheep”. *Small Rum. Res.* 2004, 52, 81-91).

### **Summary of the invention**

15

Therefore, the problem to be solved by the present invention is to discover new molecules based on hederagenin that have a persistent effect against rumen protozoa by not being hydrolysed to hederagenin thereby leading to improving ruminant growth performance, improving milk yield, reducing methane emissions from the rumen, reducing urine  
20 ammonia excretion, and/or reducing rumen acetate to propionate ratio.

The present inventors now surprisingly discovered that a small number of non-glycosylated hederagenin derivatives are able to retain their inhibitory activity against ciliate protozoa, while they also become resistant to degradation by rumen microflora.

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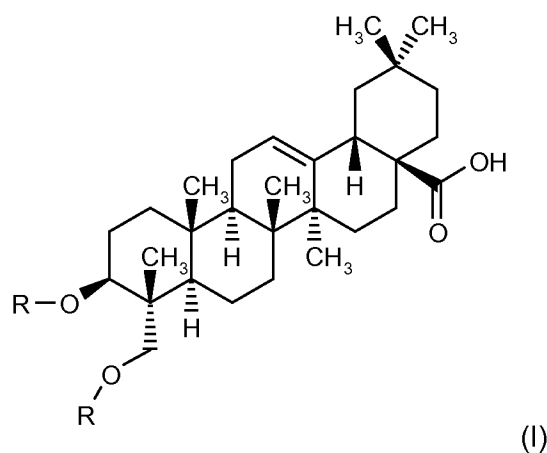
Therefore the present invention provides the use of hederagenin derivatives according to formula (I) to improve ruminant growth performance, reduce methane emission, reduce ammonia excretion, and/or to reduce rumen acetate to propionate ratio in the rumen.

30 Further aspects of the invention are subject of further independent claims. Particularly preferred embodiments are subject of dependent claims.

### **Detailed description of the invention**

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In a first aspect the present invention relates to the use of a bis ester of hederagenin of formula (I) or a salt thereof



- 5 wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

10 wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which may also be substituted with one to three hydroxy group(s),

wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which is substituted with one to four hydroxy group(s),

wherein  $R^2$  represents methyl, ethyl or propyl,

15 wherein Y represents O or  $NR^3$ ,

wherein  $R^3$  is H, methyl, ethyl or propyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ ,

20 as an active compound in ruminant animal feeding for improving growth performance, improving milk yield, reducing methane emissions from the rumen, reducing urine ammonia excretion, and/or reducing rumen acetate to propionate ratio.

In the present document, the term "bis ester" means that the two esterified R groups of the compound of formula (I) are always identical to each other. Hederagenin is a triterpenoid compound belonging to sapogenins which is a chemical constituent of the *Hedera helix*

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plant (common name "ivy"), and usually occurring in the plant in different glycosylated forms. Hederagenin is the aglycone part of the natural compound Hederagin.

The compounds of the present invention can be manufactured in principle according to synthetic methods known *per se* for esterification of organic molecules, (H.Pielartzik, B.Irmisch-Pielartzik, T.Eichler; Carbonsäureester in: Methoden der organischen Chemie (Houben-Weyl) E5 S.656-715 (1985) Stuttgart) and/or based on methods as described in the examples.

Improving ruminant performance can be assessed by methods well known in the art, and is usually characterized by feed conversion ratio, feed intake, weight gain, carcass yield, or milk yield. In a particular embodiment, improved ruminant performance means that the ruminant feed conversion ratio is reduced by at least 1 % when measured in conventional performance trials. Preferably, the feed conversion ratio is reduced by at least 2 %, more preferably, by at least 2.5 %, even more preferably, by at least 3 %, most preferably, by at least 3.5 %.

Methane emission by ruminants can easily be measured in individual animals in metabolic chambers by methods known in the art (Grainger *et al.*, 2007 J. Dairy Science; 90: 2755-2766). Moreover, it can also be assessed at barn level by an emerging technology using laser beam (McGinn *et al.*, 2009, Journal of Environmental Quality; 38: 1796-1802). Alternatively, methane produced by a dairy ruminant can also be assessed by measurement of VFA profiles in milk according to WO 2009/156453.

The amount of the compound of formula (I) fed to the ruminant animal is from 1 mg to 50 g per Kg of feed, preferably from 10 mg to 20 g per Kg of feed, more preferably, from 50 mg to 1 g per Kg of feed. For the use in animal feed, however, the compound of formula (I) need not to be that pure; it may e.g. include other compounds and derivatives.

A ruminant according to the present invention is a mammal and includes cattle, goats, sheep, giraffes, American Bison, European bison, yaks, water buffalo, deer, camels, alpacas, llamas, wildebeest, antelope, pronghorn, and nilgai. For all embodiments of the present invention, domestic cattle, sheep and goat are the more preferred species. For the present purposes most preferred species are domestic cattle. The term includes all

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breeds of domestic cattle, and all production kinds of cattle, in particular dairy cows and beef cattle.

In a preferred embodiment compounds of formula (I) are characterized in that R  
5 represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

wherein X represents a  $C_1$  to  $C_5$  linear or branched aliphatic, saturated or  
10 unsaturated hydrocarbyl group, which may also be substituted with one hydroxy group,

wherein  $R^1$  represents a  $C_1$  to  $C_5$  linear or branched aliphatic, saturated or  
unsaturated hydrocarbyl group, which is substituted with one to two hydroxy group(s),

wherein  $R^2$  represents methyl,

15 wherein Y represents O or  $NR^3$ , and  $R^3$  is H, or methyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ .

In a more preferred embodiment compounds of formula (I) are characterized in that R  
20 represents:  $C(=O)X-COOH$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ , or  $SO_3^- M^+$ ,

wherein X represents a  $C_1$  to  $C_5$  linear alkyl group, which may also be substituted  
with one hydroxy group,

wherein Y represents O or  $NR^3$ ,

25 wherein  $R^3$  is H, or methyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

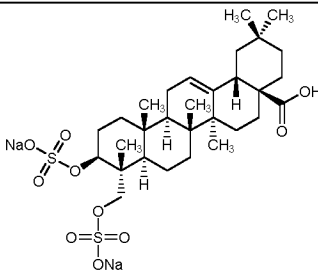
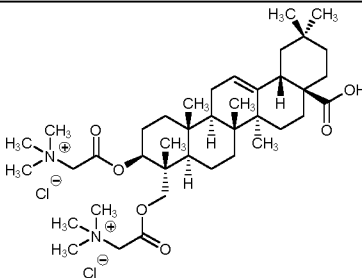
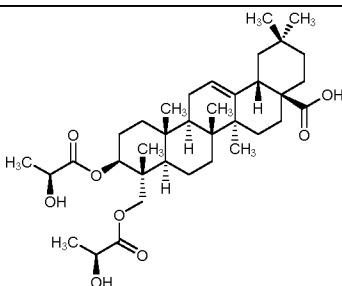
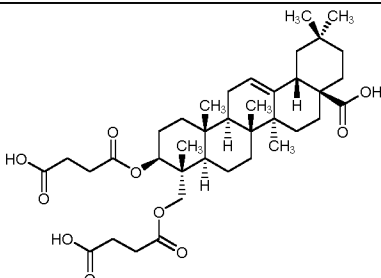
wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ .

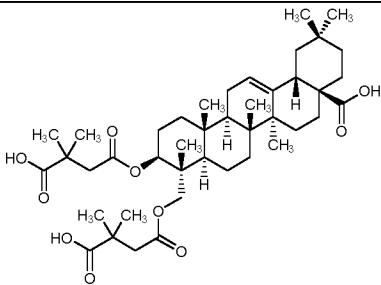
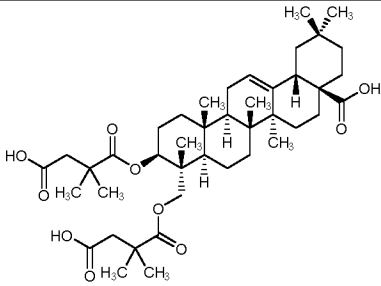
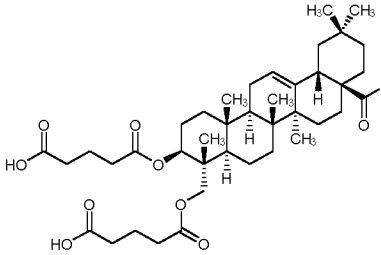
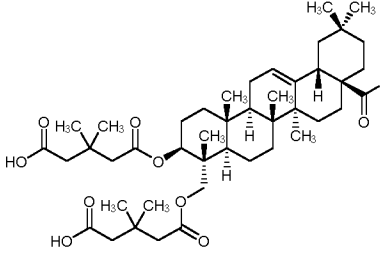
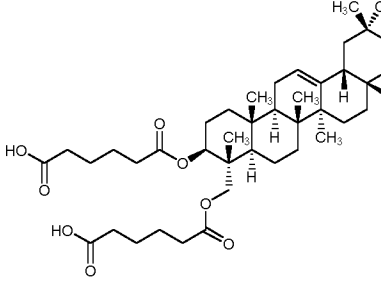
Even more preferably, for all the embodiments of the present invention, the compounds of  
30 formula (I) are selected from Hederagenin bisulfate disodium salt; Hederagenin dibetainate dichloride; Hederagenin bislactate; Hederagenin disuccinate; Hederagenin bis-4-(2,2-dimethylsuccinate); Hederagenin bis-4-(3,3-dimethylsuccinate); Hederagenin diglutarate; Hederagenin bis-(3,3-dimethylglutarate); Hederagenin diadipate; Hederagenin bis(glycin carbamate); Hederagenin bis(diglycolate); Hederagenin bis-(methylimino-

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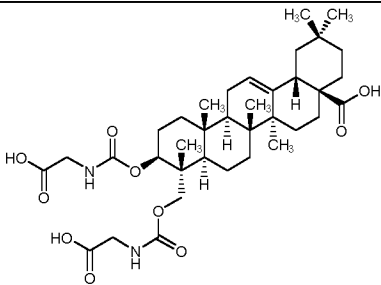
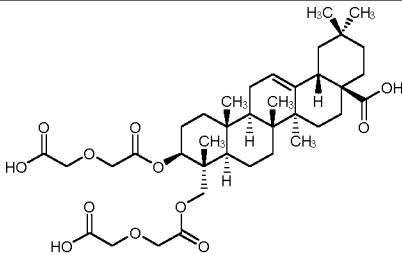
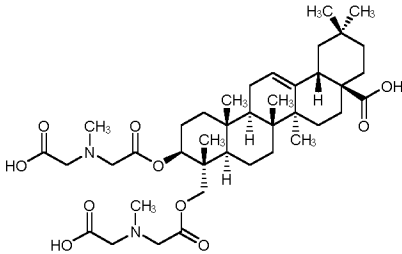
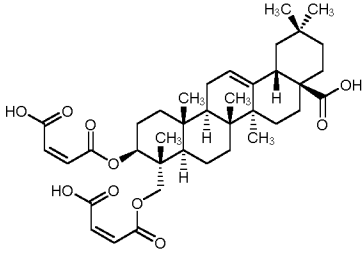
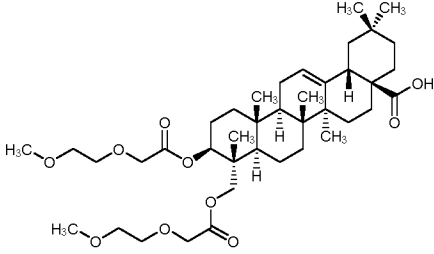
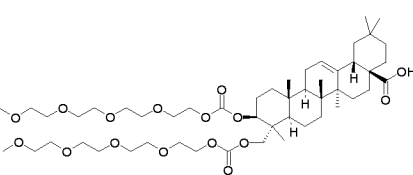
diacetate); Hederagenin dimaleate; Hederagenin bis-(2-methoxyethoxy)acetate; Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate), which structures are shown in Table 1, and any mixture thereof.

5 **Table 1:** Preferred compounds according to the present invention:

| Entry | Name                                | Structure                                                                            | code  |
|-------|-------------------------------------|--------------------------------------------------------------------------------------|-------|
| 1     | Hederagenin bisulfate disodium salt |    | TSB38 |
| 2     | Hederagenin dibetainate dichloride  |   | TSB37 |
| 3     | Hederagenin bislactate              |  | TSB44 |
| 4     | Hederagenin disuccinate             |  | TSB24 |

|   |                                           |                                                                                      |       |
|---|-------------------------------------------|--------------------------------------------------------------------------------------|-------|
| 5 | Hederagenin bis-4-(2,2-dimethylsuccinate) |    | TSB45 |
| 6 | Hederagenin bis-4-(3,3-dimethylsuccinate) |    | TSB52 |
| 7 | Hederagenin diglutarate                   |   | TSB35 |
| 8 | Hederagenin bis-(3,3-dimethylglutarate)   |  | TSB46 |
| 9 | Hederagenin diadipate                     |  | TSB47 |



|    |                                                     |                                                                                      |       |
|----|-----------------------------------------------------|--------------------------------------------------------------------------------------|-------|
| 10 | Hederagenin bis(glycin carbamate)                   |    | TSB36 |
| 11 | Hederagenin bis(diglycolate)                        |    | TSB50 |
| 12 | Hederagenin bis-(methyliminodiacetate)              |   | TSB51 |
| 13 | Hederagenin dimaleate                               |  | TSB53 |
| 14 | Hederagenin bis-(2-methoxyethoxy)acetate            |  | TSB33 |
| 15 | Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate) |  | TSB34 |

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Most preferred compounds according to the present invention are selected from Hederagenin bisulfate disodium salt; Hederagenin dibetainate dichloride; Hederagenin disuccinate; Hederagenin diglutarate, and any mixture thereof.

- 5 For the realisation of their use as such ingredients for the feed of ruminants the compounds may be incorporated in the feed by methods known per se in the art of feed formulation and processing. Therefore, in a further aspect, the present invention relates to a feed composition or a feed additive comprising at least one compound of formula (I) as described above, or a salt thereof. As indicated above, the compounds of the present
- 10 invention are useful as compounds for feed additives and animal feed compositions for ruminants, and accordingly are useful as the active ingredients in such feed to improve growth performance, improve milk yield, reduce methane emissions from the rumen, reduce urine ammonia excretion, and/or reduce rumen acetate to propionate ratio.
- 15 Preferably, the feed composition or feed additive is a ruminant base mix. In a preferred embodiment, the composition is a mineral premix, a vitamin premix including vitamins and minerals, a bolus or a lick stone.

The normal daily dosage of a compound according to the invention provided to an animal

20 by feed intake depends upon the kind of animal and its condition. Normally this dosage should be in the range of from about 1 mg to about 50 g, preferably from about 10 mg to about 20 g, more preferably, 50 mg to 1 g compound per kg of feed.

The compound of formula (I) according to the present invention may be used in

25 combination with conventional ingredients present in an animal feed composition (diet) such as calcium carbonates, electrolytes such as ammonium chloride, proteins such as soya bean meal, wheat, starch, sunflower meal, corn, meat and bone meal, amino acids, animal fat, vitamins and trace minerals.

- 30 Particular examples of compositions of the invention are the following:
- An animal feed additive comprising (a) at least one compound selected from table 1 and (b) at least one fat-soluble vitamin, (c) at least one water-soluble vitamin, (d) at least one trace mineral, and/or (e) at least one macro mineral;

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- An animal feed composition comprising at least one compound selected from table 1 and a crude protein content of 50 to 800 g/kg feed.

Therefore, in a preferred embodiment, the present invention relates to a ruminant feed  
5 composition or feed additive.

The so-called premixes are examples of animal feed additives of the invention. A premix designates a preferably uniform mixture of one or more micro-ingredients with diluents and/or carrier. Premixes are used to facilitate uniform dispersion of micro-ingredients in a  
10 larger mix.

Apart from the active ingredients of the invention, the premix of the invention contains at least one fat-soluble vitamin, and/or at least one water-soluble vitamin, and/or at least one trace mineral, and/or at least one macro mineral. In other words, the premix of the  
15 invention comprises the at least one compound according to the invention together with at least one additional component selected from the group consisting of fat-soluble vitamins, water-soluble vitamins, trace minerals, and macro minerals.

Macro minerals may be separately added to the feed. Therefore, in a particular  
20 embodiment, the premix comprises the active ingredients of the invention together with at least one additional component selected from the group consisting of fat-soluble vitamins, water-soluble vitamins, and trace-minerals.

The following are non-exclusive lists of examples of these components:

- 25 - Examples of fat-soluble vitamins are vitamin A, vitamin D3, vitamin E, and vitamin K, e.g. vitamin K3.
- Examples of water-soluble vitamins are vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, e.g. Ca-D-panthothenate.
- Examples of trace minerals are manganese, zinc, iron, copper, iodine, selenium, and  
30 cobalt.
- Examples of macro minerals are calcium, phosphorus and sodium.

As regards feed compositions for ruminants such as cows, as well as ingredients thereof, the ruminant diet is usually composed of an easily degradable fraction (named

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concentrate) and a fiber-rich less readily degradable fraction (named hay, forage, or roughage).

Hay is made of dried grass, legume or whole cereals. Grasses include among others  
5 timothy, ryegrasses, fescues. Legumes include among others clover, lucerne or alfalfa,  
peas, beans and vetches. Whole cereals include among others barley, maize (corn), oat,  
sorghum. Other forage crops include sugarcane, kales, rapes, and cabbages. Also root  
crops such as turnips, swedes, mangles, fodder beet, and sugar beet (including sugar  
beet pulp and beet molasses) are used to feed ruminants. Still further crops are tubers  
10 such as potatoes, cassava and sweet potato. Silage is an ensiled version of the fiber-rich  
fraction (e.g. from grasses, legumes or whole cereals) whereby material with a high water  
content is treated with a controlled anaerobic fermentation process (naturally-fermented or  
additive treated).

15 Concentrate is largely made up of cereals (such as barley including brewers grain and  
distillers grain, maize, wheat, sorghum), but also often contain protein-rich feed  
ingredients such as soybean, rapeseed, palm kernel, cotton seed and sunflower.  
Cows may also be fed total mixed rations (TMR), where all the dietary components, e.g.  
forage, silage and concentrate, are mixed before serving.

20

As mentioned above a premix is an example of a feed additive which may comprise the  
active compounds according to the invention. It is understood that the compounds may be  
administered to the animal in different other forms. For example the compounds can also  
be included in a bolus that would be placed in the rumen and that would release a defined  
25 amount of the active compounds continuously in well-defined dosages over a specific  
period of time.

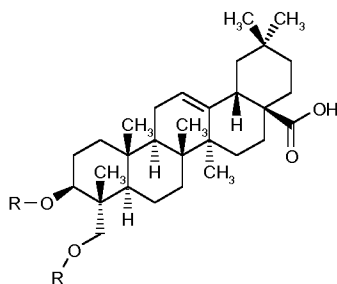
Furthermore, the compound of formula (I) may conveniently be added to a lick stone also  
called mineral lick.

30

In a further aspect, the present invention relates to a method for improving growth  
performance, improving milk yield, reducing methane emissions from the rumen, reducing  
urine ammonia excretion, and/or reducing rumen acetate to propionate ratio of a ruminant

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animal, comprising orally administering a sufficient amount of at least one active compound as defined in formula (I)



(I)

wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  
 5  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  
 $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which may also be substituted with one to three hydroxy group(s),

10 wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which is substituted with one to four hydroxy group(s),

wherein  $R^2$  represents methyl, ethyl or propyl,

wherein Y represents O or  $NR^3$ ,

15 wherein  $R^3$  is H, methyl, ethyl or propyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ ,

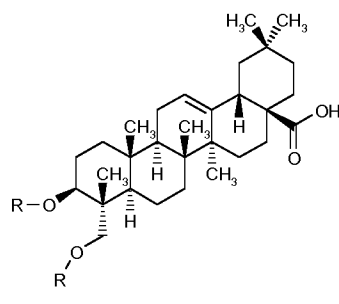
with the preferred embodiments described above.

20 Preferably, the compounds of formula (I) are selected from hederagenin, Hederagenin bisulfate disodium salt; Hederagenin dibetainate dichloride; Hederagenin disuccinate; Hederagenin diglutarate, and any mixture thereof.

In a still further aspect, the present invention relates to bis esters of hederagenin

25 according to formula (I) or a salt thereof

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wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  
 $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  
 5  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which may also be substituted with one to three hydroxy group(s),

wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or  
 10 unsaturated hydrocarbyl group, which is substituted with one to four hydroxy group(s),

wherein  $R^2$  represents methyl, ethyl or propyl,

wherein Y represents O or  $NR^3$ ,

wherein  $R^3$  is H, methyl, ethyl or propyl,

15 wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ ,

wherein the compound of formula (I) is not hederagenin disuccinate.

The present invention is further illustrated by the following experiments.

20

## Examples

### Example 1: Activity of bis esters of hederagenin in inhibiting predation of bacteria by protozoa.

25

The modified triterpene were tested for their effectiveness on rumen protozoa activity by quantifying their capacity to breakdown  $^{14}C$ -labelled rumen bacteria. The following hederagenin bis esters were tested: (TSB22: Hederagenin diacetate, TSB23: Hederagenin dibutyrate, TSB24: Hederagenin disuccinate, TSB33: Hederagenin bis-(2-

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methoxyethoxy)acetate, TSB34: Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate, TSB35: Hederagenin diglutarate, TSB36: Hederagenin bis(glycin carbamate), TSB37: Hederagenin dibetainate dichloride, TSB38: Hederagenin bisulfate disodium salt were synthesized as described in examples below.

5

## **Materials and methods**

### **Preparation of labelled bacteria**

The activity of protozoal populations was measured *in vitro* from the breakdown of  
10 [14C]labelled rumen bacteria as described by Wallace and McPherson (1987). Total rumen bacteria were isolated from rumen fluid obtained from four barren rumen-cannulated Holstein-Frisian cows fed a diet balanced to meet maintenance requirements. *Streptococcus bovis* was cultured for 4 days on medium no. 2 of Hobson (1969) at 39°C by daily transfer of culture into fresh media. The day before the experiment, bacterial  
15 cultures were isotope-labelled by growing in medium no. 2 of Hobson containing [14C]leucine (1.89 µ Ci/7.5 mL tube). On the day of the experiment labelled bacteria were harvested from the cultures, washed and re-suspended in simplex type salts solution (STS) (Williams and Coleman, 1992) containing [12C]leucine (5 mmol/L) to prevent re-incorporation of released [14C]leucine by bacteria. Re-suspended labelled bacteria were  
20 used as inoculum in the incubation and were subsampled to determine its radioactivity.

### **Preparation of the ivy "refined extract"**

Ivy fruit meal was extracted with ethanol leading to a crude extract comprising triglycerids  
25 („fats“), saponins, oligosaccharides („sugars“) and pigments (anthocyanins). The crude extract was then further extracted with petrol ether. Other solvents like hexane or diethyl ether also worked well. The resulting materials comprises mainly saponins and oligosaccharides and was called the Defatted extract. Finally, the refined extract was produced by an n-butanol extraction leading to a refined extract mainly comprising  
30 saponins.

### **Protozoal incubation**

Strained rumen fluid was diluted in STS (1:1) and incubated (7.5ml) with 14C-labelled bacteria (0.5 mL) in Hungate tubes containing the modified triterpene per quadruplicate, at

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0.1, 0.5 and 1 mg/ml. Modified triterpens were diluted in ethanol with the exception of TSB38 only soluble in DMSO. Therefore incubations with ethanol or DMSO alone were also carried out. Ivy "refined extract" (1 mg/ml) was used, in most of the cases, as a positive control (but it was not included in the statistical analysis).

5

Incubation was carried out at 39°C under CO<sub>2</sub> and tubes were sampled at time 0: 0h and at 1h intervals up to 5 h using a syringe with a 23 gauge needle. Samples (0.5 mL) were acidified (by adding 125 µL of trichloroacetic acid at 25 % wt/vol) and centrifuged (13000 × g for 5 min). Supernatant (200 µL), was diluted with 2 mL of scintillation fluid to determine the radioactivity released by liquid-scintillation spectrometry. The release of <sup>14</sup>C trichloroacetic acid soluble material during the incubation reflects degradation of the labelled bacteria and hence protozoal activity. Bacterial breakdown at each incubation time was expressed as the percentage of the acid-soluble radioactivity released in respect of the total radioactivity present in the labelled bacteria suspension.

15

#### **Statistical analysis**

Data at different time points were analysed by repeated measurements ANOVA, using inoculum as a block term. Means of treatments were compared by least significant difference (LSD) test. Statistical significance was considered if P<0.05.

20

#### **Results and discussion**

The results of this experiment are summarized in Table 2. The table shows the released activity expressed as a percentage for the different compounds at different concentrations and time points. Thus, it represents the capacity to breakdown <sup>14</sup>C-labelled rumen bacteria. The negative control (ethanol or DMSO) represents the maximum protozoa predation activity in absence of any inhibitor expressed as percentage of <sup>14</sup>C-labelled rumen bacteria released. The positive control (ivy saponins) at 1 mg/ml shows the maximal inhibition of protozoal activity when using ivy saponins.

30 Results showed that all the modified triterpens tested were effective in reducing bacterial predation by protozoa at 0.5 and 1 mg/ml (P<0.001) in comparison with the negative control. The greatest effect on protozoa was observed in the presence of Hederagenin disuccinate (TSB24), Hederagenin diglutarate (TSB35), Hederagenin dibetainate



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dichloride (TSB37), and Hederagenin bisulfate disodium salt (TSB38) which caused the complete abolition of predatory activity, when added at those concentrations.

**Table 2.**

| Time (Hours)                      | 0          | 1          | 2           | 3           | 4           | 5           |
|-----------------------------------|------------|------------|-------------|-------------|-------------|-------------|
| <b>Control (-)</b>                | <b>5.0</b> | <b>8.3</b> | <b>13.6</b> | <b>17.6</b> | <b>22.0</b> | <b>22.0</b> |
| <b>Control (+): Ivy (1 mg/ml)</b> | <b>4.0</b> | <b>3.7</b> | <b>3.9</b>  | <b>3.9</b>  | <b>4.0</b>  | <b>4.4</b>  |
| TSB24 (0.1 mg/ml)                 | 6.0        | 8.1        | 10.4        | 14.0        | 21.7        | 21.8        |
| TSB24 (0.5 mg/ml)                 | 4.8        | 5.3        | 5.8         | 5.1         | 5.1         | 5.1         |
| TSB24 (1 mg/ml)                   | 5.2        | 5.1        | 5.1         | 4.5         | 4.3         | 4.3         |
| TSB33 (0.1 mg/ml)                 | 4.4        | 6.4        | 8.2         | 10.8        | 10.8        | 14.0        |
| TSB33 (0.5 mg/ml)                 | 3.8        | 5.2        | 7.0         | 8.6         | 9.1         | 10.3        |
| TSB33 (1 mg/ml)                   | 4.1        | 4.9        | 6.4         | 7.4         | 8.4         | 8.9         |
| TSB34 (0.1 mg/ml)                 | 4.1        | 5.5        | 8.7         | 10.9        | 13.0        | 14.9        |
| TSB34 (0.5 mg/ml)                 | 4.1        | 4.6        | 5.8         | 6.8         | 8.0         | 8.6         |
| TSB34 (1 mg/ml)                   | 4.4        | 4.8        | 5.5         | 6.7         | 6.9         | 8.8         |
| TSB35 (0.1 mg/ml)                 | 4.2        | 5.8        | 8.2         | 9.7         | 11.6        | 12.8        |
| TSB35 (0.5 mg/ml)                 | 4.1        | 4.3        | 4.2         | 4.5         | 4.6         | 4.5         |
| TSB35 (1 mg/ml)                   | 3.9        | 4.4        | 5.0         | 4.8         | 4.8         | 5.0         |
| TSB36 (0.1 mg/ml)                 | 3.9        | 6.4        | 9.8         | 12.3        | 13.4        | 16.4        |
| TSB36 (0.5 mg/ml)                 | 3.9        | 5.1        | 7.3         | 8.3         | 8.6         | 9.7         |
| TSB36 (1 mg/ml)                   | 4.0        | 4.3        | 4.6         | 4.8         | 4.6         | 5.0         |
| TSB37 (0.1 mg/ml)                 | 3.7        | 5.7        | 7.9         | 10.0        | 11.5        | 13.0        |
| TSB37 (0.5 mg/ml)                 | 4.1        | 4.9        | 4.8         | 5.2         | 5.3         | 5.4         |
| TSB37 (1 mg/ml)                   | 4.3        | 4.9        | 4.8         | 5.0         | 5.0         | 5.4         |
| TSB38 (0.1 mg/ml)                 | 3.9        | 5.6        | 8.5         | 10.7        | 13.3        | 14.8        |
| TSB38 (0.5 mg/ml)                 | 3.8        | 5.1        | 6.6         | 7.3         | 8.7         | 8.9         |
| TSB38 (1 mg/ml)                   | 4.4        | 4.4        | 4.7         | 4.9         | 5.3         | 6.0         |

5

**Table 3**

| Time (Hours)           | 0    | 1    | 2     | 3     | 4     | 5     |
|------------------------|------|------|-------|-------|-------|-------|
| CONTROL (-)            | 4.41 | 7.05 | 10.25 | 13.82 | 16.59 | 19.09 |
| Control (+): Ivy 1 g/L | 4.42 | 4.68 | 4.86  | 5.23  | 5.24  | 5.72  |
| TSB53 0.05             | 4.41 | 6.86 | 10.33 | 13.78 | 16.93 | 19.56 |

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|           |      |      |       |       |       |       |
|-----------|------|------|-------|-------|-------|-------|
| TSB53 0.1 | 4.71 | 6.75 | 10.43 | 12.61 | 15.32 | 17.59 |
| TSB53 0.5 | 4.60 | 4.70 | 4.52  | 4.73  | 4.72  | 4.86  |
| TSB53 1   | 4.73 | 4.71 | 4.65  | 4.78  | 5.25  | 5.24  |

Table 4

| Time (Hours)           | 0    | 1     | 2     | 3     | 4     | 5     |
|------------------------|------|-------|-------|-------|-------|-------|
| CONTROL (-)            | 5.68 | 12.09 | 21.21 | 29.81 | 35.86 | 42.67 |
| Control (+): Ivy 1 g/L | 5.63 | 5.73  | 7.00  | 6.79  | 8.84  | 9.84  |
| TSB44 0.05             | 5.40 | 10.74 | 16.38 | 20.46 | 24.16 | 29.31 |
| TSB44 0.1              | 5.50 | 6.40  | 7.53  | 8.44  | 10.10 | 10.51 |
| TSB44 0.5              | 5.37 | 5.57  | 5.63  | 5.47  | 6.12  | 5.63  |
| TSB44 1                | 5.60 | 5.73  | 5.76  | 5.96  | 5.89  | 6.18  |
| TSB45 0.05             | 5.37 | 7.45  | 10.38 | 12.89 | 15.35 | 17.60 |
| TSB45 0.1              | 5.53 | 5.80  | 6.15  | 6.74  | 6.58  | 7.49  |
| TSB45 0.5              | 5.70 | 5.46  | 6.09  | 6.18  | 6.12  | 6.54  |
| TSB45 1                | 5.40 | 5.70  | 5.63  | 5.99  | 6.28  | 6.32  |
| TSB46 0.05             | 5.60 | 7.35  | 10.01 | 11.59 | 14.03 | 15.88 |
| TSB46 0.1              | 5.14 | 5.79  | 6.09  | 6.77  | 7.55  | 7.65  |
| TSB46 0.5              | 5.57 | 5.54  | 5.70  | 6.09  | 6.15  | 6.48  |
| TSB46 1                | 5.14 | 5.63  | 5.95  | 5.57  | 6.35  | 6.22  |
| TSB47 0.05             | 5.57 | 9.46  | 15.83 | 20.77 | 25.26 | 29.38 |
| TSB47 0.1              | 5.37 | 6.57  | 8.07  | 9.80  | 11.58 | 12.94 |
| TSB47 0.5              | 5.31 | 5.70  | 5.83  | 6.22  | 6.32  | 6.12  |
| TSB47 1                | 5.44 | 6.22  | 6.68  | 6.61  | 7.13  | 7.32  |
| TSB48 0.05             | 5.46 | 11.92 | 20.16 | 27.00 | 34.82 | 40.19 |
| TSB48 0.1              | 5.82 | 11.24 | 19.05 | 26.84 | 34.57 | 38.69 |
| TSB48 0.5              | 5.53 | 12.18 | 19.15 | 26.36 | 33.67 | 38.74 |
| TSB48 1                | 5.18 | 12.21 | 19.90 | 27.72 | 34.40 | 39.99 |
| TSB50 0.05             | 5.73 | 12.28 | 21.99 | 31.39 | 36.47 | 42.02 |
| TSB50 0.1              | 5.24 | 12.66 | 22.23 | 29.80 | 35.92 | 41.33 |
| TSB50 0.5              | 5.50 | 8.39  | 12.42 | 16.96 | 18.84 | 22.12 |
| TSB50 1                | 5.21 | 8.26  | 11.61 | 14.34 | 15.48 | 17.47 |
| TSB51 0.05             | 5.53 | 12.47 | 22.21 | 30.92 | 37.22 | 43.20 |
| TSB51 0.1              | 5.24 | 12.02 | 21.11 | 27.60 | 33.39 | 37.35 |
| TSB51 0.5              | 5.50 | 7.54  | 9.49  | 11.47 | 13.30 | 14.20 |
| TSB51 1                | 5.63 | 6.96  | 9.30  | 10.21 | 10.89 | 12.16 |
| TSB52 0.05             | 5.50 | 7.38  | 10.14 | 12.76 | 15.62 | 17.01 |

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|           |      |      |      |      |      |      |
|-----------|------|------|------|------|------|------|
| TSB52 0.1 | 5.27 | 5.82 | 5.66 | 6.47 | 6.77 | 6.57 |
| TSB52 0.5 | 5.53 | 5.47 | 5.76 | 5.86 | 5.66 | 5.76 |
| TSB52 1   | 5.37 | 5.24 | 5.95 | 5.86 | 5.63 | 5.86 |

Surprisingly, the hederagenin bis esters as shown in Table 2, 3 and 4 were as active as the original hederagenin isolated from ivy. This result is surprising, since some of the hederagenin not covered by the compound of formula (I) like hederagenin diacetate (TSB22) or hederagenin dibutyrate (TSB23) were completely inactive in this assay.

**Example 2: Activity of bis esters of hederagenin against protozoa motility over a 24 hours incubation**

The aim of this study was to test the effect of the hederagenin bis esters at 0.5 and 1 mg/ml of the incubation, on protozoa motility over time as well as on fermentation parameters after 24 h *in vitro* incubations.

Incubations of 30 ml of inoculum (SRF:buffer, 1/3:2/3) with 0.3 g of diet (barley:hay, 60:40, mix ground to pass through a 1 mm<sup>2</sup> sieve) were carried out in 120 ml wheaton bottles. Treatments consisted of: Control (-), (no saponins) and compounds of formula (I), TSB35, TSB36, and TSB37 added at 0.5 and 1 mg/ml of the incubation. Modified triterpens were added either in ethanol. For each triterpen, a 200 mg/ml solution was prepared and either 75 µl of this solution + 75 µl of ethanol (for the 0.5 mg/ml concentration) or 150 µl of the 200 µg/ml solution (for the 1 mg/ml concentration) added to the corresponding Wheatons on the day of the experiment.

Rumen fluid was collected from 4 cannulated dairy cows strained through a double layer of muslin and maintained under CO<sub>2</sub> at 39°C. Strained rumen fluid was diluted 1/3:2/3 with incubation solution (Menken and Steingass, 1988) and 30 ml were added under CO<sub>2</sub> to the corresponding Wheaton bottles and incubated at 39°C.

Samples at different time points (0, 4, 8 and 24 h) were collected for visual assessment of protozoa motility. Ciliate protozoa motility was assessed in 30 µl of sample against a common scale when examined at low magnification (x 100) using light microscopy. This evaluation was conducted very quickly (less than 1 min/sample) to avoid the cell damage originated by the oxygen and temperature exposure. A total of 10 microscope fields were

observed. A global score has been established for the protozoal population according with the following table:

**Table 5:** Scores and activity description for evaluating the protozoal activity by optical microscopy.

| Score     | Description                                                                                                                                                   |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 4.8 - 5   | High motility for all genera (>90%)                                                                                                                           |
| 4.5 - 4.7 | Medium motility for all genera                                                                                                                                |
| 4.3 - 4.5 | Low motility for all genera                                                                                                                                   |
| 4.1 - 4.2 | No motility but high ciliary activity for most protozoa. Low motility of a low percentage of present protozoa (10-20%)                                        |
| 3.5 - 4   | No motility or activity for protozoa (>60%), low ciliary activity in a low percentage of present protozoa (30-40%). Some individuals might still moving (<5%) |
| 3 - 3.4   | No motility or activity for any genera (~90%), low ciliary activity in a low percentage of present protozoa (~10%)                                            |
| 2 - 2.9   | Vacuoles visible (2= most of them; 2.9 =some of them)                                                                                                         |
| 1         | Cellular disruption (1= most of them; 1.9 =some of them)                                                                                                      |
| 0         | Not protozoa evident                                                                                                                                          |

\* Holotrich protozoa will move slightly faster than entodiniomorphid.

\*\* After 24h little or no holotrich protozoa will be present.

At the end of the experiment (24 hours), samples were collected to measure pH and analyze volatile fatty acids (VFAs) (1 ml 20% ortho-phosphoric acid containing 20 mM 2 ethyl butyric acid + 4 ml of sample) by gas liquid chromatography and ammonia analysis (1 ml sample + 0.25 µl TCA) by phenol hypochlorite method.

### **Statistical analysis**

Fermentation parameters were analyzed by ANOVA considering treatments as main factor and sheep as the block term. Fisher's unprotected least significance difference test was used to establish comparisons between treatments. Findings with  $P < 0.05$  were considered as statistically significant.

### **Results and Discussion**

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It has been reported that up to 1% of ethanol in rumen fluid should not affect fermentation, not being considered toxic to the rumen microorganisms (Aldai et al., 2012; Morgavi et al., 2004; Wallace et al., 2007). Also, our previous studies confirm this observation.

- 5 The greatest effect on protozoa motility and on fermentation parameters, was observed in the in the presence of TSB35 (Hederagenin diglutarate) and TSB37 (Hederagenin dibetainate chloride) (see Table 4.1 and 4.2) which is in agreement with the results obtained in the previous in vitro experiment with <sup>14</sup>C-labelled bacteria. These hederagenin derivatives, at the two cocentration tested, caused a decreased in the acetic/propionic ratio  
10 and ammonia concentration, as well as on protozoa motility, in comparison to the control (ethanol).

TSB34 (Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate) and TSB36 (Hederagenin bis(glycin carbamate) at the highest concentration, also decreased protozoa motility over  
15 time, although to a lesser extent, and decreased amonia concentration and acetic/propionic ratio.

**Table 6.1:**

|             | Ethanol | Ivy-0.25 | Ivy-0.5 | TSB35-0.5   | TSB35-1     | TSB36-0.5 | TSB36-1     | SED   | P      |
|-------------|---------|----------|---------|-------------|-------------|-----------|-------------|-------|--------|
| pH          | 6.31    | 6.11     | 6.12    | 6.38        | 6.38        | 6.32      | 6.29        | 0.016 | <0.001 |
| Acetic      | 53.2    | 57.6     | 57.1    | 41.7        | 38.3        | 46.1      | 44.7        | 2.853 | <0.001 |
| Propionic   | 14.7    | 18.2     | 18.5    | <b>19.2</b> | <b>20.8</b> | 14.7      | 15.6        | 1.084 | <0.001 |
| Iso-butyric | 1.19    | 1.522    | 1.2     | 0.902       | 1.135       | 1.007     | 1.01        | 0.158 | 0.67   |
| Butyric     | 9.35    | 10.1     | 9.62    | <b>5.41</b> | <b>5.01</b> | 7.97      | 6.99        | 0.519 | <0.001 |
| Iso-valeric | 0.783   | 1.312    | 1.175   | 0.613       | 0.64        | 0.648     | 0.615       | 0.05  | <0.001 |
| Valeric     | 0.905   | 1.265    | 1.28    | 0.835       | 0.848       | 0.803     | 0.753       | 0.047 | <0.001 |
| Caproic     | 0.26    | 0.353    | 0.445   | 0.243       | 0.248       | 0.24      | 0.24        | 0.015 | <0.001 |
| Ace/Pro     | 3.61    | 3.163    | 3.091   | <b>2.17</b> | <b>1.83</b> | 3.14      | <b>2.86</b> | 0.109 | <0.001 |
| TotalVFA    | 80.3    | 90.3     | 89.4    | 68.9        | 66.9        | 71.4      | 69.88       | 4.35  | 0.098  |

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|         |      |       |       |             |             |             |             |       |        |
|---------|------|-------|-------|-------------|-------------|-------------|-------------|-------|--------|
| Ammonia | 12   | 10.7  | 10.6  | <b>10.6</b> | <b>10.7</b> | <b>10.5</b> | <b>9.09</b> | 0.711 | <0.001 |
| Pro0    | 4.85 | 4.775 | 4.775 | <b>4.78</b> | <b>4.83</b> | 4.8         | <b>4.85</b> | 0.053 | 0.247  |
| Pro4    | 4.8  | 4.75  | 4.625 | <b>4.05</b> | <b>3.53</b> | 4.68        | <b>4.63</b> | 0.099 | <0.001 |
| Pro8    | 4.93 | 4.825 | 4.55  | <b>3.63</b> | <b>2.75</b> | 4.73        | <b>4.23</b> | 0.118 | <0.001 |
| Pro24   | 4.78 | 4.85  | 4.875 | <b>4.05</b> | <b>3.25</b> | 4.4         | <b>3.95</b> | 0.156 | <0.001 |

**Table 6.2:**

|             | Ethanol | Ivy-0.25 | Ivy-0.5 | TSB37-0.5   | TSB37-1     | SED   | P      |
|-------------|---------|----------|---------|-------------|-------------|-------|--------|
| pH          | 6.31    | 6.11     | 6.12    | 6.35        | 6.4         | 0.016 | <0.001 |
| Acetic      | 53.2    | 57.6     | 57.1    | 46.2        | 43          | 2.853 | <0.001 |
| Propionic   | 14.7    | 18.2     | 18.5    | <b>17.4</b> | <b>21.9</b> | 1.084 | <0.001 |
| Iso-butyric | 1.19    | 1.522    | 1.2     | 1.092       | 1.117       | 0.158 | 0.67   |
| Butyric     | 9.35    | 10.1     | 9.62    | <b>6.89</b> | <b>5.6</b>  | 0.519 | <0.001 |
| Iso-valeric | 0.783   | 1.312    | 1.175   | 0.615       | 0.588       | 0.05  | <0.001 |
| Valeric     | 0.905   | 1.265    | 1.28    | 0.845       | 0.63        | 0.047 | <0.001 |
| Caproic     | 0.26    | 0.353    | 0.445   | 0.25        | 0.233       | 0.015 | <0.001 |
| Ace/Pro     | 3.61    | 3.163    | 3.091   | <b>2.65</b> | <b>1.96</b> | 0.109 | <0.001 |
| TotalVFA    | 80.3    | 90.3     | 89.4    | 73.3        | 73          | 4.35  | 0.098  |
| Ammonia     | 12      | 10.7     | 10.6    | <b>11.3</b> | <b>8.96</b> | 0.711 | <0.001 |
| Pro0        | 4.85    | 4.775    | 4.775   | <b>4.85</b> | <b>4.78</b> | 0.053 | 0.247  |
| Pro4        | 4.8     | 4.75     | 4.625   | <b>4.48</b> | <b>4.03</b> | 0.099 | <0.001 |
| Pro8        | 4.93    | 4.825    | 4.55    | <b>4.23</b> | <b>3.75</b> | 0.118 | <0.001 |
| Pro24       | 4.78    | 4.85     | 4.875   | <b>4</b>    | <b>3.38</b> | 0.156 | <0.001 |

- 5 Table 7: Effect of different doses of hederagenin derivatives (0, 0.5 or 1 g/l) on in vitro rumen fermentation and protozoa motility over time

- 22 -

| TSB33       | Dose (g/L) |        |       | SED   | P-value   |       |       |
|-------------|------------|--------|-------|-------|-----------|-------|-------|
|             | 0          | 0.5    | 1     |       | Treatment | time  | Txt   |
| pH          | 6.31       | 6.32   | 6.31  | 0.009 | 0.824     |       |       |
| Ammonia     |            |        |       |       |           |       |       |
| mM          | 12         | 12.3   | 12    | 0.701 | 0.923     |       |       |
| Total VFA   |            |        |       |       |           |       |       |
| mM          | 80.1       | 78.6   | 76.3  | 3.88  | 0.647     |       |       |
| % Acetic    | 66.1       | 65.3   | 65.2  | 0.435 | 0.148     |       |       |
| % Propionic | 18.6a      | 16.7ab | 20.3b | 0.516 | 0.038     |       |       |
| % Butyric   | 11.7       | 11.5   | 11.1  | 0.25  | 0.1       |       |       |
| % BCVFA     | 2.47       | 2.29   | 2.29  | 0.126 | 0.307     |       |       |
| Protozoa    | 4.84       | 4.47   | 4.76  | 0.038 | 0.097     | 0.011 | 0.088 |

| TSB34       | Dose (g/L) |        |       | SED    | P-value   |        |       |
|-------------|------------|--------|-------|--------|-----------|--------|-------|
|             | 0          | 0.5    | 1     |        | Treatment | time   | Txt   |
| pH          | 6.31       | 6.31   | 6.31  | 0.0125 | 0.924     |        |       |
| Ammonia     |            |        |       |        |           |        |       |
| mM          | 12         | 11.9   | 10.3  | 0.752  | 0.103     |        |       |
| Total VFA   |            |        |       |        |           |        |       |
| mM          | 80.1       | 75.03  | 73.9  | 4.19   | 0.355     |        |       |
| % Acetic    | 66.1       | 65.4   | 65.2  | 0.575  | 0.31      |        |       |
| % Propionic | 18.6a      | 20.1ab | 20.8b | 0.715  | 0.05      |        |       |
| % Butyric   | 11.7b      | 11.2ab | 10.7a | 0.231  | 0.017     |        |       |
| % BCVFA     | 2.47b      | 2.19ab | 2.11a | 0.124  | 0.056     |        |       |
| Protozoa    | 4.84a      | 4.71b  | 4.61c | 0.039  | 0.003     | <0.001 | 0.134 |

| TSB38       | Dose (g/L) |        |        | SED     | P-value   |       |       |
|-------------|------------|--------|--------|---------|-----------|-------|-------|
|             | 0          | 0.5    | 1      |         | Treatment | time  | Txt   |
| pH          | 6.41       | 6.39   | 6.4    | 0.01349 | 0.385     |       |       |
| Ammonia     |            |        |        |         |           |       |       |
| mM          | 1.661      | 1.591  | 0.863  | 0.2249  | 0.022     |       |       |
| Total VFA   |            |        |        |         |           |       |       |
| mM          | 74.3       | 70.6   | 67.3   | 6.32    | 0.658     |       |       |
| % Acetic    | 62.3       | 62.7   | 61.8   | 0.502   | 0.259     |       |       |
| % Propionic | 20.9a      | 22.33a | 24.54b | 0.575   | 0.002     |       |       |
| % Butyric   | 12.8c      | 11.3b  | 10.2a  | 0.4     | 0.002     |       |       |
| % BCVFA     | 2.64b      | 2.42ab | 2.22a  | 0.1196  | 0.032     |       |       |
| Protozoa    | 4.76b      | 4.71b  | 4.64a  | 0.0226  | 0.005     | 0.002 | 0.271 |

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| TSB44       | Dose (g/L) |       |       | SED    | P-value   |        |       |
|-------------|------------|-------|-------|--------|-----------|--------|-------|
|             | 0          | 0.5   | 1     |        | Treatment | time   | Txt   |
| pH          | 6.03       | 6.04  | 6.04  | 0.0307 | 0.874     |        |       |
| Ammonia     |            |       |       |        |           |        |       |
| mM          | 5.12       | 5.62  | 4.29  | 0.71   | 0.246     |        |       |
| Total VFA   |            |       |       |        |           |        |       |
| mM          | 82.5       | 82    | 82    | 2.04   | 0.958     |        |       |
| % Acetic    | 64.8       | 65.4  | 65.3  | 0.964  | 0.787     |        |       |
| % Propionic | 20.1       | 19.5  | 20.5  | 1.098  | 0.632     |        |       |
| % Butyric   | 12.1b      | 11.7b | 11a   | 0.1734 | 0.003     |        |       |
| % BCFVA     | 1.947      | 2.302 | 2.072 | 0.275  | 0.469     |        |       |
| Protozoa    | 4.79b      | 4.64a | 4.56a | 0.0414 | 0.003     | <0.001 | 0.007 |

| TSB45       | Dose (g/L) |       |       | SED   | P-value   |        |        |
|-------------|------------|-------|-------|-------|-----------|--------|--------|
|             | 0          | 0.5   | 1     |       | Treatment | time   | Txt    |
| pH          | 6.03       | 6.11  | 6.12  | 0.033 | 0.051     |        |        |
| Ammonia     |            |       |       |       |           |        |        |
| mM          | 5.12b      | 3.60a | 3.87a | 0.434 | 0.027     |        |        |
| Total VFA   |            |       |       |       |           |        |        |
| mM          | 82.5       | 80.9  | 73.4  | 3.47  | 0.079     |        |        |
| % Acetic    | 64.8b      | 61.6a | 61.1a | 1.177 | 0.041     |        |        |
| % Propionic | 20.1a      | 27.2b | 28.3b | 1.44  | 0.002     |        |        |
| % Butyric   | 12.1       | 7.74  | 7.62  | 0.394 | <0.001    |        |        |
| % BCFVA     | 1.95       | 2.36  | 1.85  | 0.321 | 0.305     |        |        |
| Protozoa    | 4.79b      | 4.26a | 4.21a | 0.053 | <0.001    | <0.001 | <0.001 |

| TSB46       | Dose (g/L) |       |       | SED    | P-value   |        |        |
|-------------|------------|-------|-------|--------|-----------|--------|--------|
|             | 0          | 0.5   | 1     |        | Treatment | time   | Txt    |
| pH          | 6.16       | 6.11  | 6.1   | 0.0341 | 0.32      |        |        |
| Ammonia     |            |       |       |        |           |        |        |
| mM          | 4.5        | 3.54  | 2.7   | 0.665  | 0.092     |        |        |
| Total VFA   |            |       |       |        |           |        |        |
| mM          | 70.1       | 73.8  | 73.8  | 3.29   | 0.467     |        |        |
| % Acetic    | 64.1b      | 61.6a | 60.3a | 0.767  | 0.007     |        |        |
| % Propionic | 18.3a      | 27.4b | 28.9b | 0.984  | <0.001    |        |        |
| % Butyric   | 14.3       | 8.26  | 7.76  | 0.571  | <0.001    |        |        |
| % BCFVA     | 2.08       | 1.68  | 2.01  | 0.096  | 0.012     |        |        |
| Protozoa    | 4.78b      | 4.27a | 4.19a | 0.053  | <0.001    | <0.001 | <0.001 |

| Dose (g/L) |  |  |  | P-value |  |  |
|------------|--|--|--|---------|--|--|
|------------|--|--|--|---------|--|--|



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| TSB47           | 0     | 0.5   | 1     | SED    | Treatment | time   | Txt    |
|-----------------|-------|-------|-------|--------|-----------|--------|--------|
| pH              | 6.16  | 6.11  | 6.15  | 0.043  | 0.567     |        |        |
| Ammonia<br>mM   | 4.50b | 2.84a | 3.16a | 0.355  | 0.008     |        |        |
| Total VFA<br>mM | 70.1  | 73.1  | 70.8  | 3.16   | 0.62      |        |        |
| % Acetic        | 64.1c | 59.1b | 56.8a | 0.875  | <0.001    |        |        |
| % Propionic     | 18.3a | 30.4b | 33.7c | 1      | <0.001    |        |        |
| % Butyric       | 14.3b | 7.34a | 6.78a | 0.608  | <0.001    |        |        |
| % BCVFA         | 2.08  | 1.91  | 1.77  | 0.166  | 0.263     |        |        |
| Protozoa        | 4.78b | 3.72a | 3.38a | 0.2234 | <0.001    | <0.001 | <0.001 |

| TSB50           | Dose (g/L) |       |       | SED   | P-value   |       |       |
|-----------------|------------|-------|-------|-------|-----------|-------|-------|
|                 | 0          | 0.5   | 1     |       | Treatment | time  | Txt   |
| pH              | 6.16       | 6.1   | 6.11  | 0.036 | 0.294     |       |       |
| Ammonia<br>mM   | 4.5        | 3.08  | 3.39  | 0.664 | 0.16      |       |       |
| Total VFA<br>mM | 70.1       | 73.3  | 73.3  | 3.84  | 0.638     |       |       |
| % Acetic        | 64.1       | 64.4  | 65.2  | 1.07  | 0.582     |       |       |
| % Propionic     | 18.3a      | 20.2b | 20.5b | 0.698 | 0.041     |       |       |
| % Butyric       | 14.3b      | 12.6a | 11.7a | 0.506 | 0.005     |       |       |
| % BCVFA         | 2.08b      | 1.79a | 1.62a | 0.109 | 0.016     |       |       |
| Protozoa        | 4.78b      | 4.52a | 4.51a | 0.052 | 0.004     | 0.002 | 0.104 |

| TSB51           | Dose (g/L) |       |       | SED   | P-value   |        |      |
|-----------------|------------|-------|-------|-------|-----------|--------|------|
|                 | 0          | 0.5   | 1     |       | Treatment | time   | Txt  |
| pH              | 6.16       | 6.06  | 6.1   | 0.038 | 0.122     |        |      |
| Ammonia<br>mM   | 4.5b       | 3.80b | 2.76a | 0.313 | 0.004     |        |      |
| Total VFA<br>mM | 70.1       | 69.7  | 74    | 3.84  | 0.503     |        |      |
| % Acetic        | 64.1       | 65.6  | 64.7  | 0.77  | 0.207     |        |      |
| % Propionic     | 18.3a      | 19.8b | 22.4c | 0.496 | <0.001    |        |      |
| % Butyric       | 14.3b      | 11.7a | 10.2a | 0.606 | 0.001     |        |      |
| % BCVFA         | 2.08b      | 1.69a | 1.63a | 0.08  | 0.003     |        |      |
| Protozoa        | 4.78b      | 4.48a | 4.44a | 0.033 | <0.001    | <0.001 | 0.01 |

| TSB52 | Dose (g/L) |     |   | SED | P-value   |      |     |
|-------|------------|-----|---|-----|-----------|------|-----|
|       | 0          | 0.5 | 1 |     | Treatment | time | Txt |

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|             |       |       |       |       |        |        |      |
|-------------|-------|-------|-------|-------|--------|--------|------|
| pH          | 6.16  | 6.14  | 6.16  | 0.042 | 0.834  |        |      |
| Ammonia     |       |       |       |       |        |        |      |
| mM          | 4.5b  | 2.42a | 2.66a | 0.335 | 0.002  |        |      |
| Total VFA   |       |       |       |       |        |        |      |
| mM          | 70.1  | 69.5  | 71.3  | 4.49  | 0.914  |        |      |
| % Acetic    | 64.1b | 60.8a | 60.3a | 0.827 | 0.008  |        |      |
| % Propionic | 18.3a | 28.6b | 29.7b | 1.18  | <0.001 |        |      |
| % Butyric   | 14.3b | 7.86a | 7.33a | 0.746 | <0.001 |        |      |
| % BCVFA     | 2.08b | 1.59a | 1.58a | 0.088 | 0.002  |        |      |
| Protozoa    | 4.78c | 3.89b | 3.53a | 0.133 | <0.001 | <0.001 | 0.01 |

| TSB24       | Dose (g/L) |        |       | SED   | P-value   |        |      |
|-------------|------------|--------|-------|-------|-----------|--------|------|
|             | 0          | 0.5    | 1     |       | Treatment | time   | Txt  |
| pH          | 6.16       | 6.11   | 6.15  | 0.039 | 0.493     |        |      |
| Ammonia     |            |        |       |       |           |        |      |
| mM          | 4.5        | 4.5    | 3.28  | 0.493 | 0.077     |        |      |
| Total VFA   |            |        |       |       |           |        |      |
| mM          | 70.1       | 65.4   | 70.9  | 4.39  | 0.448     |        |      |
| % Acetic    | 64.1b      | 62.1b  | 59.4a | 0.837 | 0.004     |        |      |
| % Propionic | 18.3a      | 25.9b  | 30.5c | 1.13  | <0.001    |        |      |
| % Butyric   | 14.3c      | 8.75b  | 7.15a | 0.606 | <0.001    |        |      |
| % BCVFA     | 2.08b      | 1.96ab | 1.81a | 0.08  | 0.045     |        |      |
| Protozoa    | 4.78c      | 4.35b  | 3.14a | 0.052 | <0.001    | <0.001 | 0.01 |

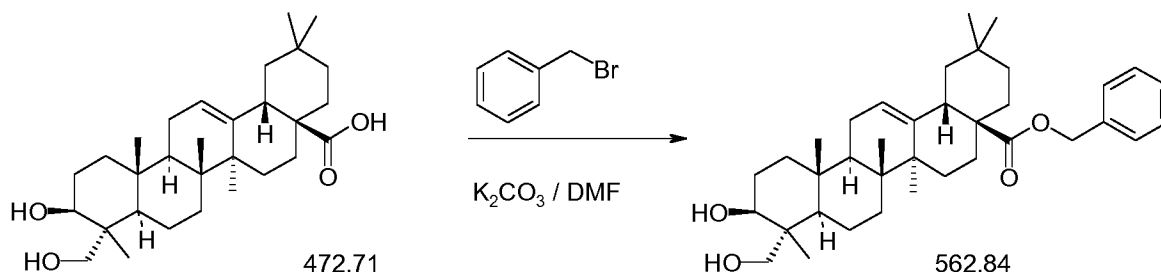
### **Example 3: Synthesis of bis esters of Hederagenin.**

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#### **3.1 Preparation of raw materials**

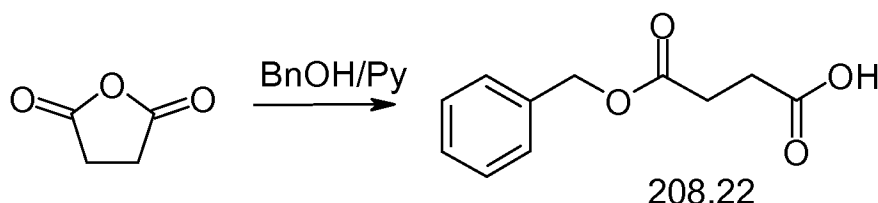
##### **a) Hederagenin benzyl ester (CAS 219550-95-5)**

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5.13 g (10 mmol) of Hederagenin (92%), 10 ml of DMF (dimethyl formamide), 2.79 g (20 mmol) of potassium carbonate and 2.62 g (15 mmol) of benzyl bromide were combined and stirred for 4 hours at ambient temperature. The reaction mixture was diluted with water and extracted with diethyl ether and concentrated in the vacuum. The product was purified by chromatography on silica gel to result in 5.0 g of Hederagenin benzyl ester.

10 **b) Succinic acid monobenzyl ester (CAS 103-40-2)**

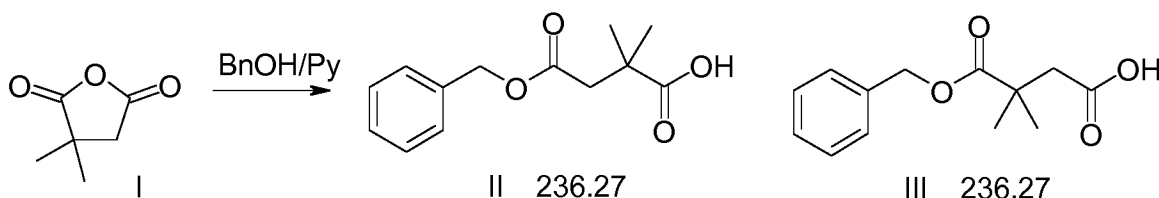


10.0 g (100 mmol) of succinic anhydride, 10 ml of THF (tetrahydrofuran), 10.8 g (100 mmol) of benzyl alcohol and 4 ml of pyridine were combined and stirred for 5 days at ambient temperature. The solution was diluted with toluene. The mixture was extracted with a saturated solution of sodium hydrogen carbonate. The water phase was separated, and acidified with hydrochloric acid. The product was extracted with toluene to yield 18.5 g of succinic acid monobenzyl ester.

20

**c) 2,2-Dimethylsuccinic acid monobenzyl ester (CAS 85216-67-7);  
3,3-dimethylsuccinic acid-4-benzylester**

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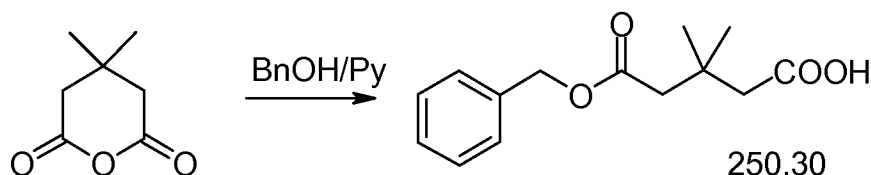


3.37 g (25 mmol) of 2,2-Dimethylsuccinic anhydride, 3.0 g (28 mmol) of benzyl alcohol, 3 ml of THF and 1 ml of pyridine were stirred for 4 days at ambient temperature. The solution was diluted with diethyl ether and the products were extracted with a saturated solution of sodium hydrogencarbonate. The water phase was separated and acidified with hydrochloric acid. The mixture of the esters was extracted with diethyl ether. The organic phase was evaporated to give 5.8 g of a colourless oil containing the mixture of the two isomeric esters II and III.

The major ester II crystallised from cyclohexane at ambient temperature. The crystallisate was filtered, and dried in the vacuum to give 3.1 g of 2,2-dimethylsuccinic acid-4-benzylester II (CAS 85216-67-7).

The mother liquor was evaporated and dried to give 2.5 g of 3,3-dimethylsuccinic acid-4-benzylester III (containing 60% ester III).

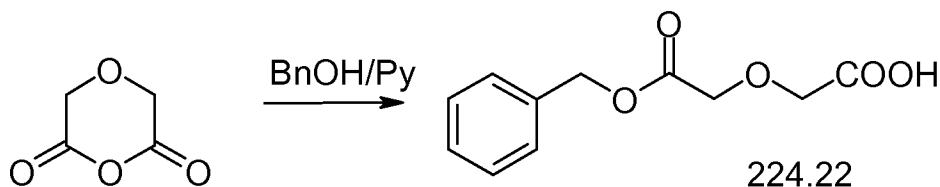
**d) 3,3-Dimethylglutaric acid monobenzyl ester (CAS 90393-31-0)**



A similar procedure as for the manufacture of succinic acid monobenzyl ester described in example 3.1 b) was used to obtain 3,3-dimethylglutaric acid monobenzyl ester.

**e) Diglycolic acid monobenzyl ester (CAS 154741-21-6)**

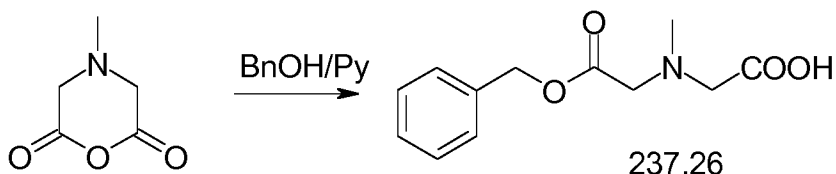
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3.25 g (25 mmol) of Diglycolic anhydride, 2.95 g (27 mmol) of benzyl alcohol, 5 ml of toluene and 1 ml of pyridine were combined. The mixture was stirred for 2 hours at  
 5 ambient temperature.

The mixture was diluted with diethyl ether and extracted with a saturated solution of sodium hydrogencarbonate. The water phase was separated and acidified by hydrochloric acid. The product was extracted with diethyl ether to yield 5.08 g of diglycolic acid  
 10 monobenzyl ester.

**f) N-Methyliminodiacetic acid monobenzyl ester**



15 3.24 g (24 mmol) of N-Methylmorpholine-2,6-dione, 4.47 g (27 mmol) of benzyl alcohol, 5 ml of tetrahydrofuran and 1 ml of pyridine were combined. The mixture was stirred for 2 days at ambient temperature.

20 The mixture was diluted with water and washed with diethyl ether. The water phase was evaporated and dried. The residue was stirred in dichloromethane for 24 hours. The suspension was filtered. The filtrate was evaporated and dried to result in 4.7 g of N-methyliminodiacetic acid monobenzyl ester.

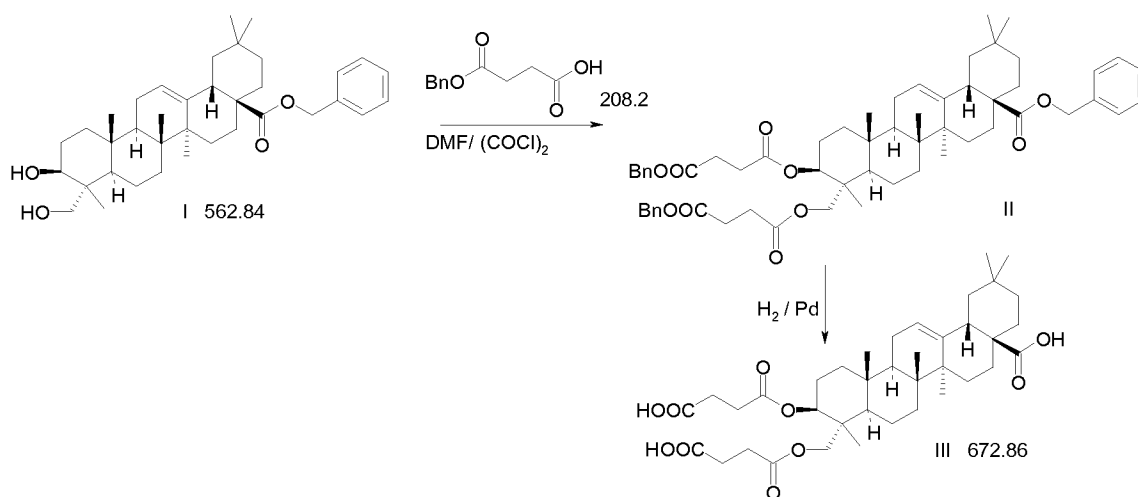
25 **3.2 Preparation of hederagenin esters:**

**a) Hederagenin disuccinate (CAS1396816-91-3) (Method A)**

- 29 -

0.70 ml (8 mmol) of Oxalyl chloride in 2 ml of acetonitrile was added to a solution of 3 ml of dimethylformamide and 3 ml of acetonitrile at -20°C. The resulting suspension was stirred for 20 minutes at -20°C. A solution of 1.67 g of succinic acid monobenzyl-ester (8 mmol) in 4 ml of acetonitrile was added with a syringe. The solution was stirred for 30 minutes at 0°C. At -20°C a solution of 1.13 g of hederagenin benzyl ester (2 mmol) in 4 ml of pyridine (50 mmol) was added. The mixture was stirred for 30 minutes at 0°C.

The mixture was diluted with diethyl ether and acidified with 1 mol/l hydrochloric acid. The mixture was extracted with diethyl ether and washed with water. The organic phase was evaporated and purified by chromatography on a column with silica gel to obtain 1.4 g of benzyl ester II.



1.14 g of benzyl ester II, 10 ml of THF (tetrahydrofuran) and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred for 18 hours in an atmosphere of hydrogen at ambient temperature. The catalyst was filtered off. The filtrate was evaporated and dried in the vacuum to result in 0.98 g of hederagenin disuccinate as whitish foam (Content = 84%).

#### **b) Hederagenin diadipate (Method A)**

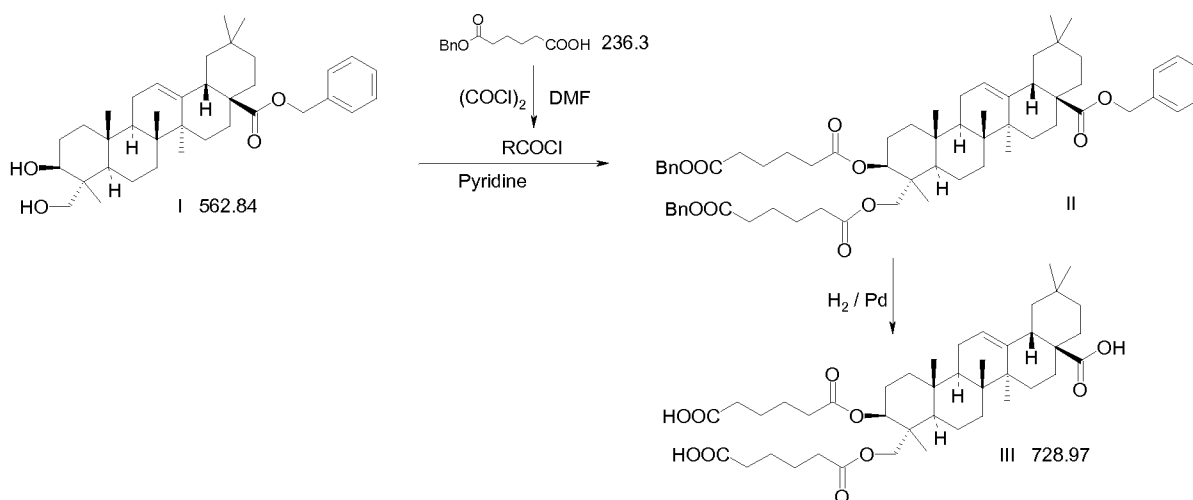
20

A similar procedure as for the manufacture of Hederagenin disuccinate was applied: To a solution of 1.5 ml of dimethylformamide and 1.5 ml of acetonitrile was added at -20°C a solution of 0.35 ml of oxalyl chloride (4 mmol) in 2 ml of acetonitrile. The slurry was stirred for 20 minutes at -20°C, followed by the addition of 1.68 g of adipic acid monobenzyl ester (4 mmol) in 2 ml of acetonitrile. The solution was stirred for 30 minutes at 0°C, followed by

25

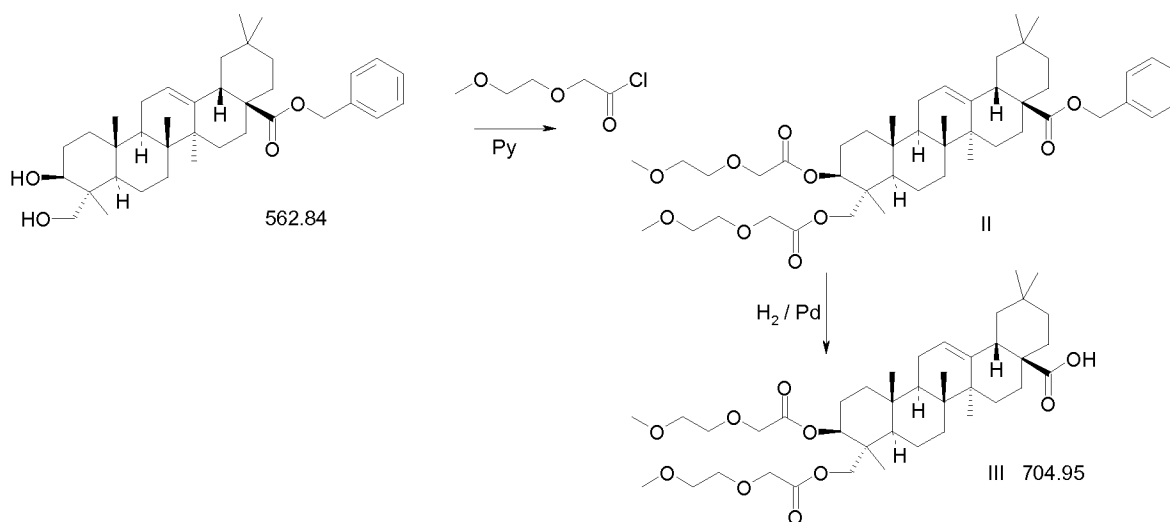
- 30 -

the addition of a solution of 0.58 g of hederagenin benzyl ester (1 mmol) in 2 ml of pyridine at  $-20^{\circ}\text{C}$ . The slurry was stirred for 1 hour at  $-20^{\circ}\text{C}$ . The mixture was diluted with diethyl ether and acidified with 0.5 mol/l hydrochloric acid. The organic phase was washed with water and evaporated. The residue was purified by chromatography on silica gel to yield 0.71 g of II as viscous oil.



0.71 g of Benzyl ester II, 5 ml of THF (tetrahydrofuran) and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred for 6 hours in an atmosphere of hydrogen at ambient temperature. The catalyst was filtered off and the filtrate was evaporated and dried to yield 0.52 g of Hederagenin diadipate III as colourless foam (Content= 92%).

### **c) Hederagenin bis-(2-methoxyethoxy)acetate (Method B)**



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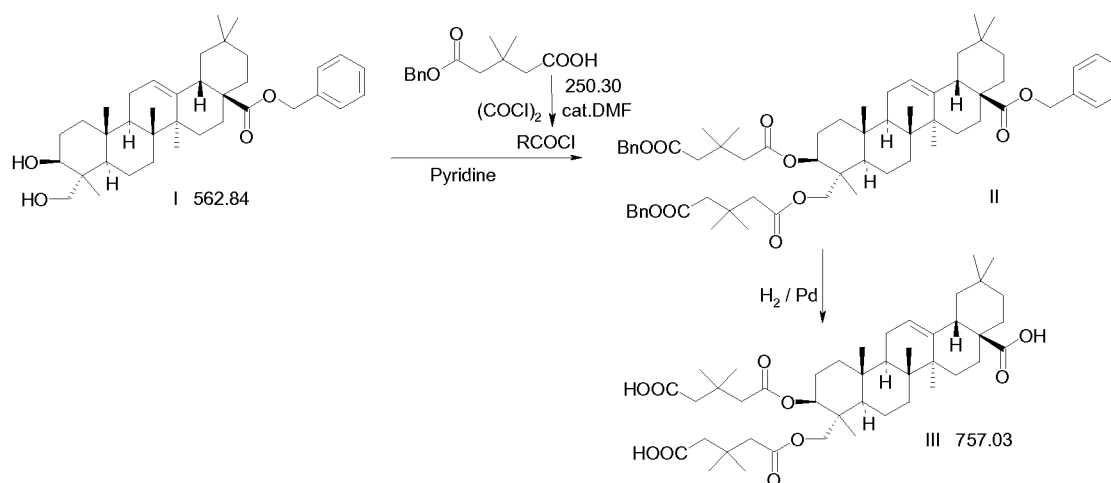
0.59 g of Hederagenin-benzyl ester (1 mmol), 10 ml of pyridine and 0.63 g of 2-(2-methoxyethoxy)acetyl chloride (4 mmol) were combined at 0°C and stirred for 2 hours at ambient temperature. The mixture was concentrated in the vacuum. The residue was purified by chromatography on silica gel to yield 0.63 g of benzyl ester II.

5

0.63 g of benzyl ester II, 6 ml of tetrahydrofuran and 50 mg of palladium 10% on charcoal (Fluka 75990) were stirred under a blanket of hydrogen for 18 hours at ambient temperature. The catalyst was filtered. The filtrate was evaporated and dried in the vacuum to result in 0.51 g of Hederagenin bis-(2-methoxyethoxy)acetate as whitish foam (content = 94%).

10

**d) Hederagenin bis-(3,3-dimethylglutarate) (Method C)**



15

To a solution of 1.03 g of 3,3-Dimethylglutaric acid monobenzenyl ester (4 mmol), 0.2 ml of 1 M DMF (dimethylformamide) in dichloromethane (0.2 mmol) and 3 ml of dichloromethane 1.8 ml of oxalyl chloride (21 mmol) was added at 1°C. The mixture was stirred for 15 minutes at 15°C. The solvent and the excess of reagents were evaporated in the vacuum.

20

The residue was diluted with 3 ml of toluene. To the solution a solution of 0.58 g of hederagenin benzyl ester (1.03 mmol) in 2 ml of pyridine was added at 0°C. The mixture was stirred for 2 hours at 0°C. The slurry was diluted with diethyl ether and acidified with 0.5 mol/l hydrochloric acid. The product was extracted with diethyl ether and washed with water. The organic phase was evaporated and the residue was purified by

25

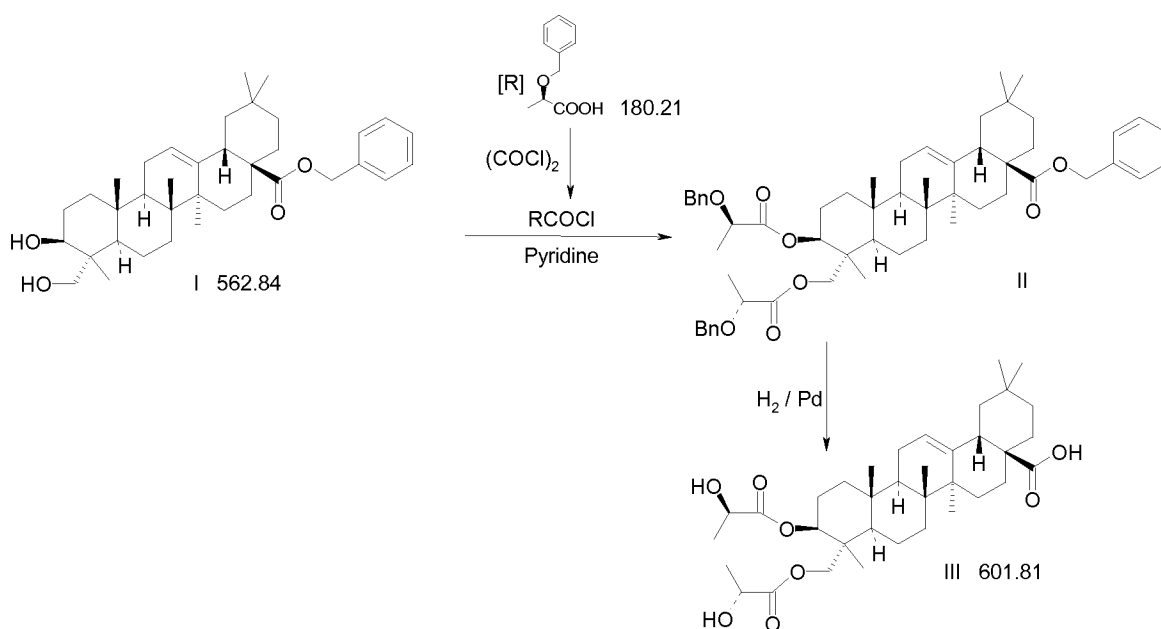
chromatography on silica gel to yield 0.93 g of benzyl ester II.



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0.92 g of Benzyl ester II, 5 ml of THF (tetrahydrofuran) and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred at ambient temperature in an atmosphere of hydrogen until complete conversion. The catalyst was filtered. The filtrate was evaporated and dried  
 5 in the vacuum to obtain 0.66 g of Hederagenin bis-(3,3-dimethylglutarate) III as colourless foam (content= 84%).

**e)Hederagenin bislactate (Method C)**



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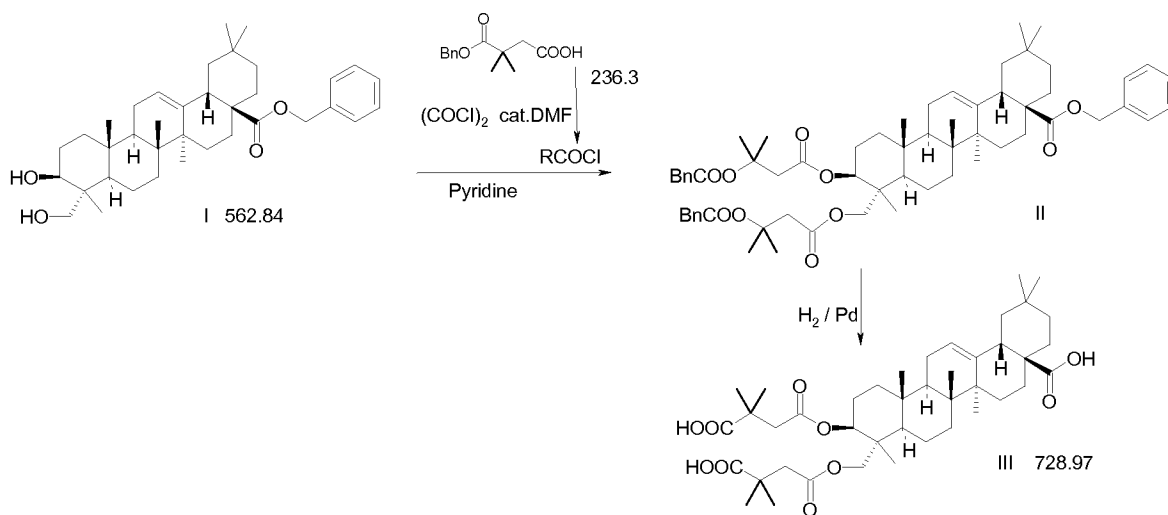
A similar process as for the manufacture of Hederagenin bis-(3,3-dimethylglutarate) was carried out: To a solution of 0.74 g of [R]-2-(benzyloxy)propanoic acid (4 mmol), 0.2 ml of 1 M DMF (dimethylformamide) in dichloromethane (0.2 mmol) and 2 ml of  
 15 dichloromethane 1.8 ml (21 mmol) of oxalyl chloride was added at 1°C. The mixture was stirred for 30 minutes at 15°C. The solvent and the excess of reagents were evaporated in the vacuum. The residue was diluted with 4 ml of toluene. To the solution a solution of 0.58 g of hederagenin benzyl ester (1.03 mmol) in 2 ml of pyridine was added at 0°C. The mixture was stirred for 2 hours at 0°C. The slurry was diluted with diethyl ether and  
 20 acidified with 0.5mol/l hydrochloric acid. The product was extracted with diethyl ether and washed with water. The organic phase was evaporated and the residue purified by chromatography on silica gel to yield 0.88 g of benzyl ester II.

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0.87 g of Benzyl ester II, 5 ml of THF (tetrahydrofuran) and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere of hydrogen at ambient temperature until complete conversion. The catalyst was filtered. The filtrate was evaporated and dried (1 mbar, 60°C) to yield 0.58 g of hederagenin bis lactate III as colourless foam (content = 92%).

**f) Hederagenin bis-4-(2,2-dimethylsuccinate) (Method C)**

A similar process as for the manufacture of Hederagenin bis-(3,3-dimethylglutarate) was carried out: To a solution of 1.41 g (3.5 mmol) 3,3-dimethylsuccinic acid-4-benzylester (60%), 1.0 ml 0.2 M DMF (dimethylformamide) in dichloromethane (0.2 mmol) and 2 ml of dichloromethane 1.8 ml (21 mmol) of oxalyl chloride was added at 1°C. The mixture was stirred for 30 minutes at 15°C. The solvent and the excess of reagents were evaporated in the vacuum. The residue was diluted with 4 ml of toluene. To the solution a solution of 0.58 g of hederagenin benzyl ester (1.0 mmol) in 2 ml of pyridine was added at 0°C. The mixture was stirred for 1 hour at 0°C. The slurry was diluted with diethyl ether and acidified with 0.5mol/l hydrochloric acid. The water phase was separated. The organic phases were washed with water. The organic phase was evaporated and the residue was purified by chromatography on a column with silica gel to yield 0.97 g of benzyl ester II.



0.97 g of Benzyl ester II, 5 ml of tetrahydrofuran and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere of hydrogen at ambient temperature until complete turnover. The catalyst was filtered. The filtrate was evaporated and dried in

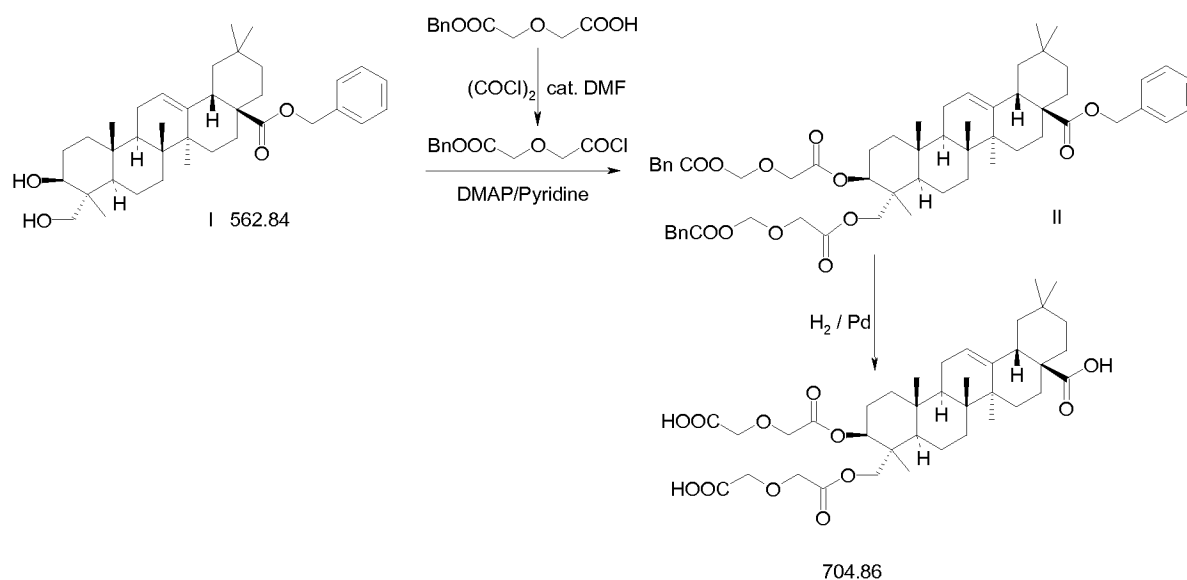
- 34 -

the vacuum to yield 0.72 g of hederagenin-di-4-(2,2-dimethylsuccinate) as colourless foam (content= 84%).

**g) Hederagenin bis(diglycolate) (Method C)**

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A similar process as for the manufacture of Hederagenin bis-(3,3-dimethylglutarate) was carried out:



- 10 To a solution of 0.96 g (4.3 mmol) of oxodiacetic acid monobenzyl ester, 1 ml of 0.2 M (0.2 mmol) dimethylformamide in dichloromethane and 3 ml dichloromethane 1.8 ml (21 mmol) of oxalyl chloride was added at 1°C. The mixture was stirred for 30 minutes at 15°C. The solvent and the excess of reagents were evaporated. The residue was diluted with 4 ml of toluene. To the solution a solution of 0.58 g of hederagenin benzyl ester (1.03 mmol) in 2 ml of pyridine was added at 0°C. The mixture was stirred for 2 hours at 0°C (TLC (thin layer chromatography): complete conversion).
- 15

The slurry was diluted with diethyl ether and acidified with 0.5 mol/l hydrochloric acid. The product was extracted with diethyl ether and washed with water. The organic phase was evaporated and the residue was purified by chromatography on silica gel to yield 0.91 g of benzyl ester II.

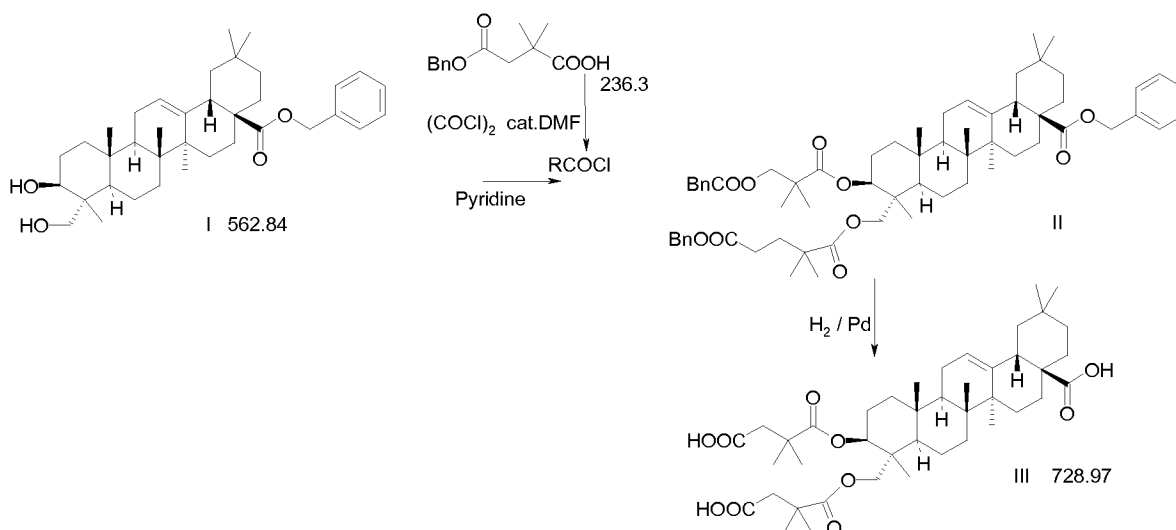
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0.91 g of Benzyl ester II, 5 ml of tetrahydrofuran and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred for 6 hours in an atmosphere of hydrogen until

- 35 -

complete conversion at ambient temperature. The reaction mixture was filtered. The filtrate was evaporated and dried in the vacuum to yield 0.64 g of hederagenin bis-diglycolate III as whitish foam (content = 90%).

5 **h) Hederagenin bis-4-(3,3-dimethylsuccinate) (Method D)**



To a solution of 0.95 g (4 mmol) of 2,2-dimethylsuccinic acid-4-benzylester, 0.5 ml of 0.2 M (0.1 mmol) DMF in dichloromethane and 2 ml dichloromethane 1.8 ml oxalyl chloride (21 mmol) was added at 1°C. The mixture was stirred for 30 minutes at 15°C. The solvent and the excess of reagents were evaporated in the vacuum. The residue was diluted with 4 ml of toluene. To the solution a solution of 0.58 g of hederagenin benzyl ester (1.03 mmol) in 2 ml of pyridine and 0.26 g of dimethylaminopyridine were added at 0°C. The suspension was stirred for 2 days at 45°C.

The slurry was diluted with diethyl ether and acidified with 0.5mol/l hydrochloric acid. The organic phases were separated and washed with water. The organic phase was evaporated and the residue was purified by chromatography on silica gel to yield 0.75 g of benzyl ester II.

0.75 g of Benzyl ester II, 5 ml of tetrahydrofuran and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere of hydrogen at ambient temperature until complete turnover. The catalyst was filtered. The filtrate was evaporated and dried in

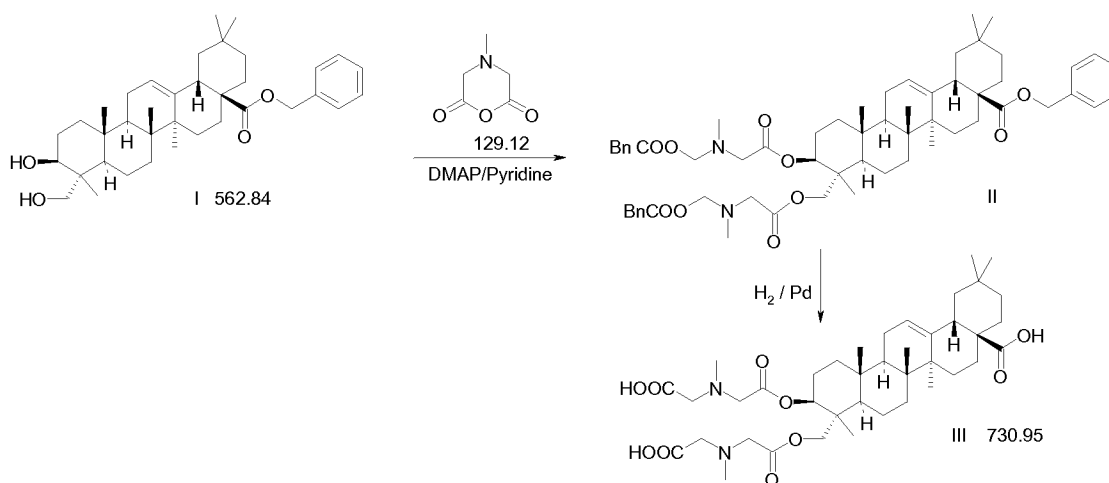
- 36 -

the vacuum to yield 0.54 g of hederagenin-di-4-(3,3-dimethylsuccinate) as colourless foam (content = 84%).

**i) Hederagenin bis-(methyliminodiacetate) (Method E)**

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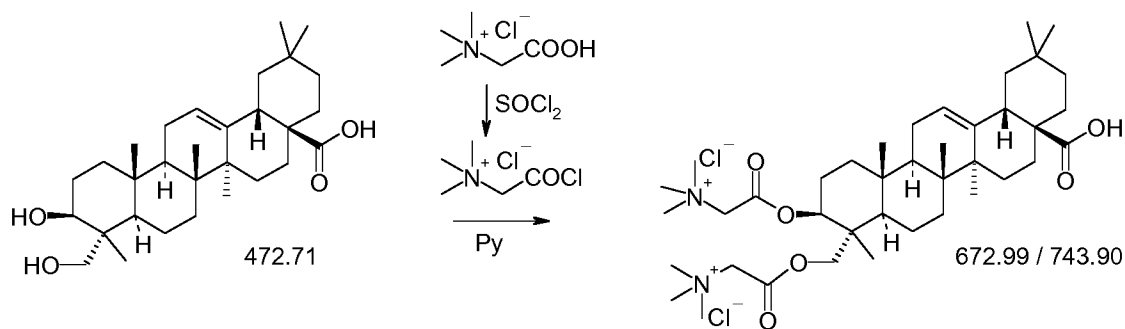
1.75 g (13.6 mmol) of 4-Methylmorpholine-2,6-dione, 0.57 g (1.0 mmol) of hederagenin benzyl ester, 10 ml of pyridine and 0.25 g of 4-dimethylaminopyridine were combined. The solution was stirred for 18 hours at ambient temperature until complete conversion. The mixture was concentrated in the vacuum. The residue was purified by chromatography on  
10 a column with reversed phase silica RP-18 to yield 0.51 g of benzyl ester II.



0.51 g of Benzyl ester II, 5 ml of tetrahydrofuran, 0.6 ml of acetic acid and 200 mg of  
15 palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere of hydrogen at ambient temperature until complete conversion. The reaction mixture was diluted with 10 ml of a mixture of acetonitrile and water (50% : 50%) and filtered. The filtrate was evaporated in the vacuum. The residue was suspended in acetonitrile, filtered and dried to yield 0.37 g of hederagenin bis-(methyliminodiacetate) III as whitish foam (content =  
20 79%).

**j) Hederagenin dibetainate chloride (Method F)**

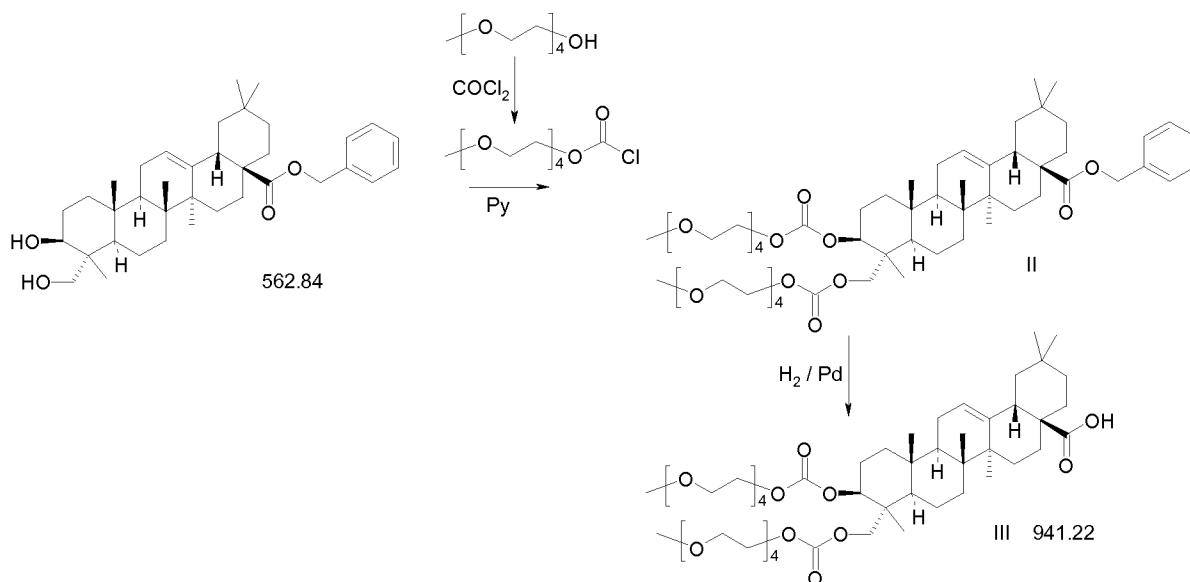
- 37 -



A mixture of 0.78 g (5 mmol) of betain chloride, 0.80 ml (11 mmol) of thionylchloride and 7 ml of toluene were stirred for 4 hours at 80°C. The solvent was evaporated. To the solid residue 0.52 g of hederagenin (1.1 mmol), 2 ml of pyridine and 2 ml of dichloromethane were added at ambient temperature. The mixture was stirred for 4 hours at ambient temperature, diluted with 25 ml of acetonitrile and filtered.

The filtrate was evaporated. The residue was slurried for 1 hour in 30 ml of acetonitrile. The suspension was filtered and dried to yield 0.54 g of Hederagenin dibetainate dichloride (content = 66%).

**k) Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate) (Method G)**



- 38 -

4.0 ml (7.6 mmol) of phosgene (20% in toluene) and 0.83 g (4 mmol) of tetraethylenglycol monomethyl ether were combined at 0°C and stirred for 1 hour at 0°C and 16 hours at ambient temperature. The solution was concentrated in the vacuum and diluted with 4 ml of toluene. To the solution 0.59 g of hederagenin benzyl ester (1 mmol) and 1 ml of  
5 pyridine were added. The mixture was stirred for 3 hours at ambient temperature and concentrated in the vacuum. The residue was purified by chromatography on silica gel to yield 0.8 g of benzyl ester II.

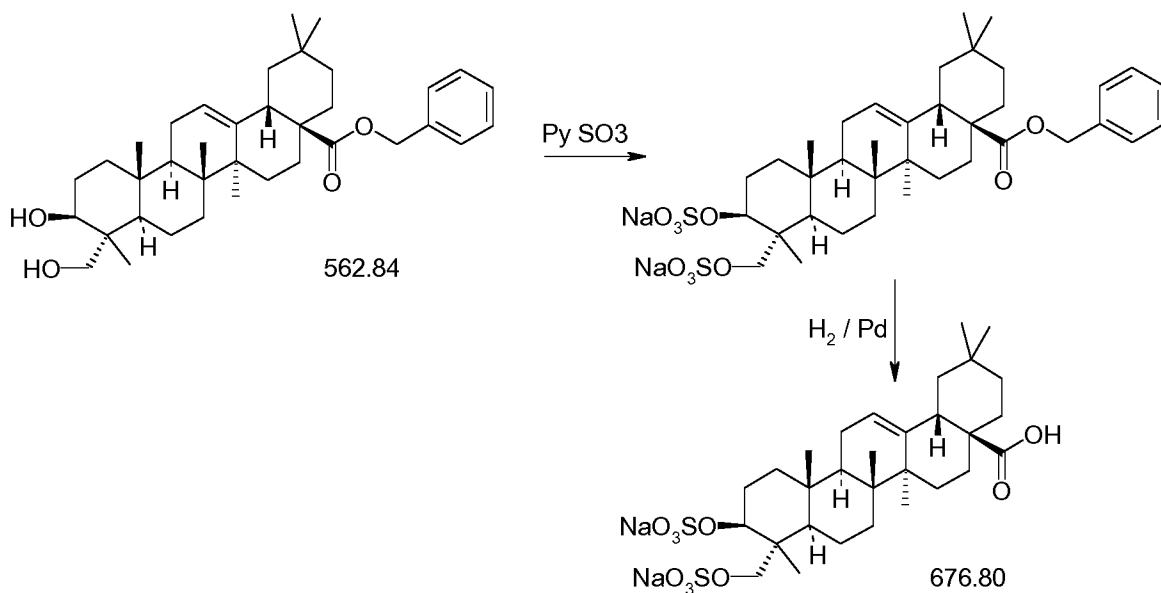
0.80 g of Benzyl ester II, 6 ml of tetrahydrofuran and 70 mg of palladium 10% on charcoal  
10 (Fluka 75990) were stirred for 18 hours in an atmosphere of hydrogen at ambient temperature. The catalyst was filtered off and the filtrate was evaporated and dried in the vacuum to yield 0.68 g of III as colourless honey (content = 95%).

**I) Hederagenin-bisulfate disodium salt (Method H)**

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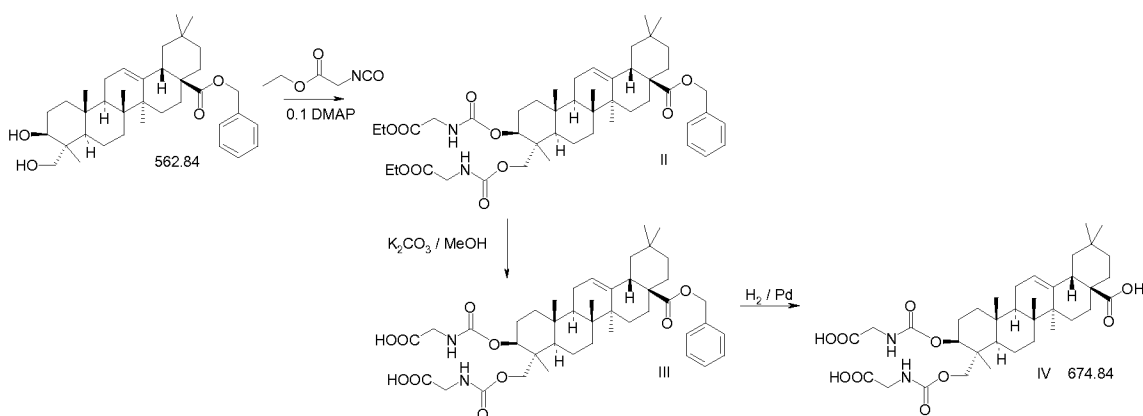
0.28 g (0.5 mmol) of Hederagenin benzyl ester, 20 ml of tetrahydrofuran, 0.5 ml of pyridine, 0.24 g of sulfurtrioxide-pyridine complex (1.4 mmol) were combined and stirred for 3 hours at ambient temperature. The mixture was evaporated and the residue solubilised in 10 ml of water. The pH was adjusted to pH = 10 with 1 mol/l of sodium  
20 hydroxide in water. The product was precipitated by addition of sodium chloride. The solids were filtered and dried in the vacuum to yield 0.52 g of hederagenin benzyl ester bisulfate disodium salt

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0.51 g of hederagenin benzyl ester bi-sulfate disodium salt, 10 ml of isopropanol, 2 ml of  
 5 water, 0.05 g of palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere  
 of hydrogen until complete conversion. The catalyst was removed and the filtrate  
 evaporated to yield 0.44 g of disodium hederagenin bi-sulfate as whitish solid (content =  
 58%).

10 **m) Hederagenin bis(glycine carbamate) (Method I)**



0.56 g (1 mmol) of Hederagenin benzyl ester, 5 ml of toluene, 0.59 ml (5 mmol) of  
 15 ethylisocyanato acetate and 13 mg (0.1 mmol) of dimethylaminopyridine were stirred for 1



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day at 50°C under a blanket of nitrogen. The mixture was purified by chromatography on silica gel to yield 0.75 g of II.

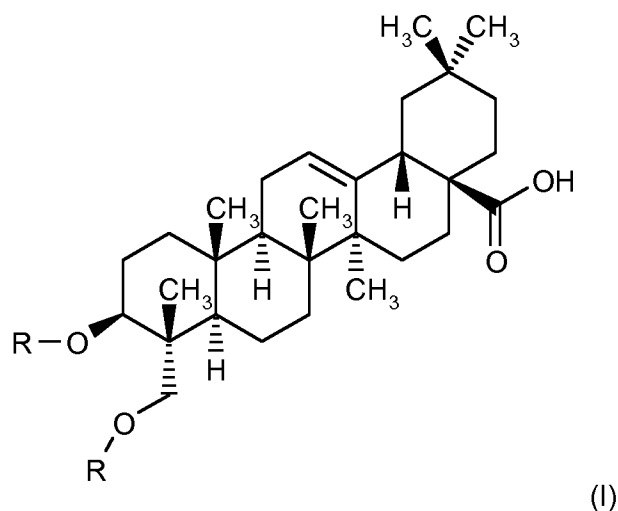
To a solution of 1.01 g (1.2 mmol) of II in 10 ml of methanol 7.5 ml of 1 M potassium carbonate (7.5 mmol) in water were added and stirred for 18 hours. The mixture was acidified with hydrochloric acid 1 mol/l and extracted with diethyl ether. The organic phases were evaporated and the residue was purified by chromatography on silica gel to yield 0.74 g of hederagenin benzyl ester dicarbamate III.

0.69 g of hederagenin benzyl ester dicarbamate III, 6 ml of tetrahydrofuran and 100 mg of palladium 10% on charcoal (Fluka 75990) were stirred in an atmosphere of hydrogen at ambient temperature for 4 hours. The mixture was filtered and evaporated to yield 0.60 g of Hederagenin-bis-glycin carbamate IV as greyish foam (content = 84%).

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**Claims**

1. Use of a bis ester of hederagenin of formula (I) or a salt thereof



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wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

10

wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbonyl group, which may also be substituted with one to three hydroxy group(s),

wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbonyl group, which is substituted with one to four hydroxy group(s),

15

wherein  $R^2$  represents methyl, ethyl or propyl,

wherein Y represents O or  $NR^3$ ,

wherein  $R^3$  is H, methyl, ethyl or propyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ ,

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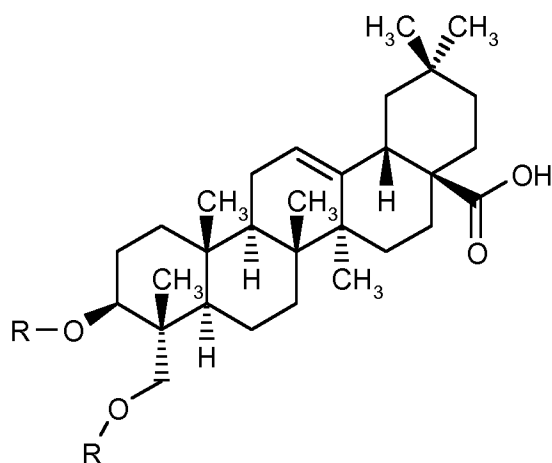
as an active compound in ruminant animal feeding for improving growth performance, improving milk yield, reducing methane emissions from the rumen, reducing urine ammonia excretion, and/or reducing rumen acetate to propionate ratio.

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2. The use of a bis ester of hederagenin of formula (I) or a salt thereof according to claim 1,  
wherein X represents a C<sub>1</sub> to C<sub>5</sub> linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which may also be substituted with one hydroxy group,  
5 wherein R<sup>1</sup> represents a C<sub>1</sub> to C<sub>5</sub> linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which is substituted with one to two hydroxy group(s),  
wherein R<sup>2</sup> represents methyl, and  
10 wherein R<sup>3</sup> is H, or methyl.
3. The use of a bis ester of hederagenin of formula (I) or a salt thereof according to claim 1 or 2, wherein R represents:  
C(=O)X-COOH, C(=O)-CH<sub>2</sub>-Y-CH<sub>2</sub>-COOH, SO<sub>3</sub><sup>-</sup> M<sup>+</sup>, or C(=O)(CH<sub>2</sub>)<sub>1-5</sub>N<sup>+</sup>(R<sup>3</sup>)<sub>3</sub>A<sup>-</sup>,  
15 wherein X represents a C<sub>1</sub> to C<sub>5</sub> linear alkyl group, which may also be substituted with one hydroxy group, and  
wherein R<sup>3</sup> is H, or methyl.
4. The use of a bis ester of hederagenin of formula (I) or a salt thereof according to claim 1, wherein, the compound of formula (I) is selected from: Hederagenin  
20 bisulfate disodium salt; Hederagenin dibetainate dichloride; Hederagenin bislactate; Hederagenin disuccinate; Hederagenin bis-4-(2,2-dimethylsuccinate); Hederagenin bis-4-(3,3-dimethylsuccinate); Hederagenin diglutarate; Hederagenin bis-(3,3-dimethylglutarate); Hederagenin diadipate; Hederagenin bis(glycin carbamate);  
25 Hederagenin bis(diglycolate); Hederagenin bis-(methyliminodiacetate); Hederagenin dimaleate; Hederagenin bis-(2-methoxyethoxy)acetate; Hederagenin di-(2,5,8,11,14-pentaoxapentadecanoate)), and any mixture thereof.
5. The use of a bis ester of hederagenin of formula (I) or a salt thereof according to claim 1, wherein the compound of formula (I) is selected from: Hederagenin bisulfate  
30 disodium salt; Hederagenin dibetainate dichloride; Hederagenin disuccinate; Hederagenin diglutarate, and any mixture thereof.

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6. A feed composition or a feed additive comprising at least one compound of formula (I) as depicted in any one or more of claims 1 to 5, or a salt thereof.
7. The composition of claim 6, which is a mineral premix, a vitamin premix, a premix including vitamins and minerals, a bolus, or a lick stone.
8. Method for improving growth performance, improving milk yield, reducing methane emissions from the rumen, reducing urine ammonia excretion, and/or reducing rumen acetate to propionate ratio of a ruminant animal, comprising orally administering a sufficient amount of at least one active compound as defined in formula (I)



(I)

wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,

wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which may also be substituted with one to three hydroxy group(s),

wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbyl group, which is substituted with one to four hydroxy group(s),

wherein  $R^2$  represents methyl, ethyl or propyl,

wherein Y represents O or  $NR^3$ , and  $R^3$  is H, methyl, ethyl or propyl,

wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,

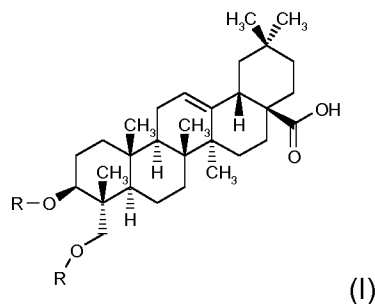
wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ .

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9. A method according to claim 8, wherein the compound of formula (I) is selected from: Hederagenin bisulfate disodium salt; Hederagenin dibetainate dichloride; Hederagenin disuccinate; Hederagenin diglutarate, and any mixture thereof.

5

10. A bis ester of hederagenin according to formula (I) or a salt thereof



- wherein R represents:  $C(=O)X-COOH$ ,  $C(=O)R^1$ ,  $C(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  
 10  $OC(=O)(CH_2)_{1-3}(OCH_2-CH_2)_{1-10}OR^2$ ,  $C(=O)-CH_2-Y-CH_2-COOH$ ,  $C(=O)NH(CH_2)_{1-3}COOH$ ,  $SO_3^- M^+$ , or  $C(=O)(CH_2)_{1-5}N^+(R^3)_3A^-$ ,  
 wherein X represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbonyl group, which may also be substituted with one to three hydroxy group(s),  
 15 wherein  $R^1$  represents a  $C_1$  to  $C_{10}$  linear or branched aliphatic, saturated or unsaturated hydrocarbonyl group, which is substituted with one to four hydroxy group(s),  
 wherein  $R^2$  represents methyl, ethyl or propyl,  
 wherein Y represents O or  $NR^3$ , and  $R^3$  is H, methyl, ethyl or propyl,  
 20 wherein  $M^+$  is a cation selected from  $Na^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $K^+$ ,  $\frac{1}{2} Mg^{2+}$ ,  $NH_4^+$ ,  
 wherein  $A^-$  is an anion selected from  $Cl^-$ ,  $Br^-$ ,  $\frac{1}{2} SO_4^{2-}$ ,  $NO_3^-$ ,  $CH_3COO^-$ ,  
 wherein the compound of formula (I) is not hederagenin disuccinate.

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2016/062383

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C07J63/00 A23K20/105 A23K50/10  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
A23K A61P C07J C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, FSTA, WPI Data, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                            | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | WO 2013/174245 A1 (GUANGZHOU BOJI MEDICINE SERVICES CO LTD [CN])<br>28 November 2013 (2013-11-28)                             | 6,7                   |
| A         | Embodiments 1, 2, 3, 4;<br>page 4<br>Embodiment 7;<br>page 5<br>Embodiments 8, 9;<br>page 6                                   | 1-5,8-10              |
| A         | -----<br>EP 0 767 177 A1 (NIPPON SHINYAKU CO LTD [JP]) 9 April 1997 (1997-04-09)<br>page 3, line 14 - line 46<br>-----<br>-/- | 1-10                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search

2 August 2016

Date of mailing of the international search report

09/08/2016

Name and mailing address of the ISA/

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Vermeulen, Stéphane

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/062383

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                        |                       |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                     | Relevant to claim No. |
| A                                                    | HART K J ET AL: "Plant extracts to manipulate rumen fermentation", ANIMAL FEED SCIENCE AND TECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 147, no. 1-3, 14 November 2008 (2008-11-14), pages 8-35, XP025506757, ISSN: 0377-8401, DOI: 10.1016/J.ANIFEEDSCI.2007.09.007 [retrieved on 2007-10-26] cited in the application page 22, paragraph 3.2 - page 28, paragraph 3.5; table 2<br>----- | 1-10                  |
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| A                                                    | BEAUCHEMIN K A ET AL: "Nutritional management for enteric methane abatement: a review", AUSTRALIAN JOURNAL OF EXPERIMENTAL AGRICULTURE, vol. 48, no. 1-2, 2008, pages 21-27, XP002750310, ISSN: 0816-1089 Plant secondary compounds; page 24, right-hand column table 2<br>-----                                                                                                       | 1-10                  |
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| A                                                    | US 2007/071849 A1 (MCNEFF LARRY C [US]) 29 March 2007 (2007-03-29) paragraph [0015] - paragraph [0020] paragraph [0027] - paragraph [0029]<br>-----                                                                                                                                                                                                                                    | 1-10                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/062383

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                                                                                                                                   | Publication<br>date                                                                                                                      |
|-------------------------------------------|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| WO 2013174245 A1                          | 28-11-2013          | CN 102659905 A<br>WO 2013174245 A1                                                                                                                           | 12-09-2012<br>28-11-2013                                                                                                                 |
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| US 2007071849 A1                          | 29-03-2007          | NONE                                                                                                                                                         |                                                                                                                                          |