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Conflict of interest statement

The authors declare a potential conflict of interest and state it below.

Authors declare that the use of synthetic bis esters of ivy saponins (bis-esters of hederagenin) in ruminants to improve ruminant growth performance, reduce methane emission, reduce urine ammonia excretion, and/or to reduce rumen acetate to propionate ratio has been patented (patent application PCT/EP2016062383)

Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure

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Abstract

The antiprotozoal effect of saponins is transitory, as when saponins are deglycosylated to the sapogenin by rumen microorganisms they become inactive. We postulated that the substitution of the sugar moiety of the saponin with small polar residues would produce sapogen-like analogues which might be resistant to degradation in the rumen as they would not be enzymatically cleaved, allowing the antiprotozoal effect to persist over time. In this study we used an acute assay based on the ability of protozoa to break down [^{14}C] leucine-labelled *Streptococcus bovis* and a longer term assay based on protozoal motility over 24 h to evaluate both the antiprotozoal effect and the stability of this effect with fifteen hederagenin *bis*-esters esterified with two identical groups, and five cholesterol and cholic acid based derivatives carrying one to three succinate residues. The acute antiprotozoal effect of hederagenin derivatives was more pronounced than that of cholesterol and cholic acid derivatives. Modifications in the structure of hederagenin, cholesterol, and cholic acid derivatives resulted in compounds with different biological activities in terms of acute effect and stability, although those which were highly toxic to protozoa were not always the most stable over time. Most of the hederagenin *bis*-esters, and in particular hederagenin *bis*-sucinate (TSB24), hederagenin *bis*-betainate dichloride (TSB37) and hederagenin *bis*-adipate (TSB47) had a persistent effect against rumen protozoa *in vitro*, shifting the fermentation pattern towards higher propionate and lower butyrate. These chemically modified triterpenes could potentially be used in ruminant diets as an effective defaunation agent to, ultimately, increase nitrogen utilization, decrease methane emissions, and enhance animal production. Further trials *in vivo* or in long term rumen simulators are now needed to confirm the *in vitro* observations presented.

Keywords: antiprotozoal activity, *Hedera helix*, hederagenin, saponins, stability

1. Introduction

The manipulation of the rumen microbial ecosystem using plant secondary compounds has proved to be a useful strategy to increase the efficiency of feed utilization by ruminants (Bodas et al., 2012; Wanapat et al., 2012). Plants or their extracts with high concentrations of saponins appear to have the potential to act as natural antiprotozoal agents (Patra and Saxena, 2009a). Protozoa are a normal but non-vital part of the rumen microbiome and can contribute up to 50% of the bio-mass in the rumen (Williams and Coleman, 1992). Because of their predation activity, rumen protozoa have been shown to be highly active in the turnover of bacterial protein in the rumen (Wallace and McPherson, 1987). Moreover, protozoa have been proven to harbour an active population of methanogenic archaea both on their external and internal surfaces (Finlay et al., 1994; Newbold et al., 1995). A recent meta-analysis has shown that the elimination of protozoa from the rumen could increase microbial protein supply to the host by up to 30% and reduce methane production by up to 11% (Newbold et al., 2015).

Saponins are plant secondary metabolites which consist of one or more sugar moieties glycosidically linked to a less polar aglycone or sapogenin (Francis et al., 2002). The sugar portion is generally made up of common monosaccharides, such as D-glucose, D-galactose, D-glucuronic acid, D-xylose, L-rhamnose, and various pentoses which are glycosidically linked as linear or branched oligosaccharides to the sapogenin. Saponins can be broadly classified based on their sapogenin structure as either triterpenoid or steroid saponins (Wina et al., 2005). The presence of different substituents in the sapogenin such as hydroxyl, hydroxymethyl, carboxyl and acyl groups, as well as differences in the composition, linkage and number of sugar chains accounts for significant structural variation and thus their bioactivity (Patra and Saxena, 2009b; Podolak et al., 2010).

Saponins can form irreversible complexes with cholesterol in the protozoal cell membrane causing cell rupture and lysis (Wina et al., 2005). Rumen protozoal species seems to differ in their sensitivity to saponins due to differences in the sterol composition of their cellular membranes leading to the suggestion that feeding saponins might lead to partial defaunation (Patra and Saxena, 2009a). The antiprotozoal effect of saponins is, however, transitory as when saponins are deglycosylated by rumen microorganisms to the sapogenin they become inactive (Newbold et al., 1997; Patra and Saxena, 2009a) which represents a challenge to their practical application in ruminant nutrition. We hypothesized that the substitution of the sugar moiety of the saponin with small polar residues would produce sapogen-like analogues which might be resistant to degradation in the rumen as they would not be enzymatically cleaved, allowing the antiprotozoal effect to persist over time. The aim of this study was to evaluate both the acute anti-protozoal action and the stability of the antiprotozoal effect of chemically synthesised hederagenin, cholesterol, and cholic acid derivatives *in vitro.*

2. Material and Methods

2.1. Hederagenin, cholesterol and cholic acid derivatives

Ripe ivy (*Hedera helix*) fruits were collected from several locations around Bangor (44.8036° N, 68.7703° W, UK), dried at 50ºC for two days and milled. Ivy fruit meal (3.79 kg) was extracted with ethanol (15 L) for 6 h, leading to a crude extract (541 g) comprising triglycerides, saponins, oligosaccharides and pigments (anthocyanins). The crude extract was then washed with petroleum ether (3 x 500 mL) and dried overnight at 50ºC under vacuum, obtaining a fine powder (368 g) which comprised mainly mixed saponins and

oligosaccharides. Then an additional extraction with n-butanol was carried out, obtaining a refined extract comprising saponins (15% DM). Hederagenin, the aglycone part of the saponins, was obtained via hydrolysis of ivy fruit refined extract in ethanolic solution with aqueous HCl.

Hederoside B, the major saponin present in the fruit extract, was obtained by gravity chromatography (Fluorochem, silica gel 40-60, $CHCl_3$/MeOH/H_2O; 90:9:1 → 75:22.5:2.5) of the defatted fruit extract. Fractions containing hederoside B were concentrated and subsequently washed with methanol. Nuclear magnetic resonance data (pyridine-d_5) of the obtained compound was in agreement with that reported in the literature (Kizu et al., 1985).

Hederagenin *bis*-esters derivatives (two identical ester moieties at position 3 and 23; Figure 1) were synthesised from the aglycone hederagenin produced above as described in patent application PCT/EP2016062383 (Ramos-Morales et al., 2016).

Cholesterol and cholic acid derivatives (Figure 2) were synthesised following the same methods for esterification of organic molecules, described in patent PCT/EP2016062383 (Ramos-Morales et al., 2016). Hederagenin, cholesterol, and cholic acid derivatives were produced by DSM Nutritional Products and Bangor University.

The purity of the synthesised compounds was established by quantitative nuclear magnetic resonance (qNMR) spectroscopy using a Bruker Ultrashielded 400 spectrometer (Bruker Corporation, Coventry, UK) confirming purities of 80 - 99% for most derivatives except TSB37 and TSB38 which had a purity of 66% and 58%, respectively. It should be noted that the antiprotozoal activity of compounds TSB37 and TSB38 may be either over or indeed underestimated due to the impurities present.

2.2. Measurement of protozoal activity

122 The effect of hederagenin, cholesterol, and cholic acid derivatives on protozoal activity
123 was measured *in vitro* as the breakdown of [^{14}C] labelled bacteria by rumen protozoa as
124 described by Wallace and McPherson (1987). Isotope-labelled bacteria were obtained by
125 growing *Streptococcus bovis* in Wallace and McPherson media (Wallace and McPherson,
126 1987) containing [^{14}C] leucine (1.89 μCi/7.5 mL tube) as the sole nitrogen source, for 24 h.
127 Cultures were centrifuged (3,000g, 15 min), supernatant discarded and pellets re-suspended
128 in 7 mL of simplex type salt solution (STS; Williams and Coleman, 1992) containing non-
129 labelled leucine (^{12}C-leucine, 5 mM). This process was repeated three times to prevent re-
130 incorporation of released [^{14}C] leucine by bacteria. The labelled bacterial suspension was
131 sampled to determine its radioactivity and then it was used as the substrate in the incubations
132 with rumen fluid.
133 Rumen digesta was obtained from four rumen-cannulated Holstein-Frisian cows (4
134 replicates), fed at maintenance level (diet composed of perennial ryegrass hay and
135 concentrate at 67:33 on DM basis). Animal procedures were carried out in accordance with
136 the Animal Scientific Procedures Act 1986 and protocols were approved by the Aberystwyth
137 University Ethical Committee. Rumen digesta was obtained before the morning feeding and
138 strained through two layers of muslin and diluted with STS (1:1). Diluted rumen fluid (7.5
139 mL) was then incubated with labelled bacteria (0.5 mL) in tubes containing no additive
140 (control) or 0.05, 0.1, 0.5 or 1 g/L of the modified triterpenes or steroids; hederoside B, a
141 natural saponin isolated from ivy fruit, was also incubated at 0.05, 0.1, 0.5 and 1 g/L.
142 Hederagenin *bis*-sulfate disodium salt (TSB38), cholesteryl succinate (TSB39) and
143 lithocholic acid succinate (TSB42) were dissolved in dimethyl sulfoxide (DMSO) at 1% of
144 the incubation volume. The rest of the derivatives and Hederoside B were solubilized in
145 ethanol at 1% of the incubation volume as it has been shown that such concentration of
146 ethanol in rumen fluid should not impair fermentation (Morgavi et al., 2004; Wallace et al.,

147 2007). Two control treatments with 1% of either DMSO or ethanol were also included in the
148 experimental design. Incubations were carried out at 39ºC under a stream of CO_2 and tubes
149 were sampled at time 0 and at 1 h intervals up to 5 h using a syringe with a 19 gauge needle.
150 Samples (0.5 mL) were acidified (by adding 125 µL of 25% (wt/vol) trichloroacetic acid and
151 centrifuged (13,000 × *g* for 5 min). Supernatant (200 µL), was diluted with 2 mL of
152 scintillation fluid to determine the radioactivity released by liquid-scintillation spectrometry
153 (Hidex 300 SL, Lablogic Systems Ltd, Broomhill, UK). Bacterial breakdown at each
154 incubation time was expressed as the percentage of the acid-soluble radioactivity released
155 relative to the total radioactivity present in the initial labelled bacteria (Wallace and
156 McPherson, 1987).
157
158 **2.3. In vitro batch cultures**
159
160 The initial protozoal population in the inoculum used in the incubations was
161 quantified by optical microscope using the procedure described by Dehority (1993) and
162 adapted by de la Fuente et al. (2006). Within the total population (5.34 log cells/mL), 65%
163 were *Entodinium*, 8% Epidinium, 21% *Diplodinium*, 3% *Isotricha* and 3% *Dasytricha*.
164 To estimate the stability of the antiprotozoal effect and measure the influence of the
165 modified triterpene and steroids on fermentation parameters, strained rumen fluid from each
166 cow was diluted 1:2 in artificial saliva solution (Menke and Steingass, 1988). Aliquots (30
167 mL) of the diluted strained rumen fluid were added anaerobically to 120 mL serum bottles
168 (Sigma-Aldrich Ltd, Dorset, UK) containing 0.3 g of diet composed of ryegrass hay and
169 barley (40:60), previously ground to pass through a 1-mm^2 mesh screen. Treatments
170 consisted of control incubations (0.3 g of diet only), with either ethanol or DMSO added at
171 1%, and incubations with the synthesised compounds (diluted in ethanol or DMSO, as

previously described) at 0.5 or 1 g/L of the incubation. To compare the antiprotozoal effect of the synthesised compounds against that of a natural saponin from ivy, hederoside B (dissolved in ethanol) was incubated at 1 g/L. Bottles were incubated at 39 ºC under CO_2 receiving a gentle mix before every sampling time. 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 in less than 1 min/sample to avoid the cell damage originated by the oxygen and temperature exposure. A score between 0 (no whole protozoa evident) and 5 (all genera active) was given according to the scale described by Newbold (2010). Fermentation pattern, in terms of pH and VFA was determined after 24 h of the incubation. A subsample (4 mL) was diluted with 1 mL of deproteinising solution (200 mL/L orthophosphoric acid containing 20 mmol/L of 2-ethylbutyric acid as an internal standard) for the determination of VFA using gas chromatography (Stewart and Duncan, 1985).

2.4. Calculations and statistical analysis

A simple linear regression was conducted to model the relationship between the percentage of radioactivity released (relative to the ^{14}C-bacterial inoculum) and the time (from 0 h to 5 h), as well as its correlation coefficient. The slope of this trend-line indicated the bacterial degradation rate (as % h^{-1}) by the rumen protozoa and ultimately their activity. Trend line slopes as well as fermentation parameters were analysed statistically by randomized block ANOVA, with individual cows as a blocking term. Inhibition of protozoa activity (% with respect to the control) was analysed using ANOVA with treatment, dose and

their interaction as fixed effects and cow as blocking term. When significant effects were detected across the different doses, means were compared by Fisher's unprotected LSD test.

Protozoal motility was analysed as a Repeated Measures Design, with treatment as main factor and incubation time as subject factor. A stability index, to estimate the persistence of the saponin effect over time, was calculated as the percentage of the motility at 8 h that remained at 24 h. Interaction between treatment and time as a measure of differential temporal dynamics between treatments was also considered. Differences were declared significant at $P<0.05$ and considered as tendencies towards significance at $P<0.10$. Genstat 15th Edition (VSN International, Hemel Hempstead, UK) was used.

3. Results

3.1. Acute anti-protozoal activity

The amount of bacteria degraded by protozoa increased linearly ($R^2 > 0.99$) over the 5 h of incubation with both control treatments (with ethanol or with DMSO). For each derivative, the rate of bacterial degradation at different doses as compared with the control is shown in supplemental Table S1. The inhibition of protozoa activity (Table 1) was significantly different between compounds and doses ($P<0.001$). Derivatives TSB44, TSB45, TSB46, TSB47, TSB52 and TSB42 were more effective in inhibiting protozoa activity than hederoside B, the major ivy saponin. Among the cholesterol and cholic acid derivatives, TSB39, TSB40 and TSB43 were less effective against protozoa than the natural saponin ($P<0.001$).

3.2. Stability of the antiprotozoal effect and effect on fermentation parameters

Based on the observed effects of the synthesised compounds on bacterial breakdown by protozoa, the two highest doses of these derivatives (0.5 and 1 g/L) and hederoside B at 1 g/L, were tested over 24 h in *in vitro* incubations. Protozoa motility over time was assessed and fermentation parameters were determined after 24 h of incubation. Due to the number of compounds tested, the experiment was carried out in different batches and hence the slightly different values for fermentation parameters between control incubations. To overcome this issue, we have compared the effects of each compound against the control run with the same batch of rumen fluid.

Cell motility, measured as an index of protozoa viability, remained unaltered (score of 4.8) over the 24 h incubation period in control incubations with ethanol or DMSO (Figures 3 and 4). The effect of hederagenin derivatives when added at 0.5 g/L or 1 g/L is shown in Figures 3a and 3b, respectively. Although, 1 g/L of hederoside B decreased protozoa motility at 4 and 8 h of the incubation (with scores of 3.88 and 3.20, respectively), there was a strong treatment x time interaction ($P=0.05$), and protozoal motility recovered afterwards (reaching a score of 4.26 at 24 h), suggesting the expected degradation of the saponin during the incubation. Some of the derivatives, TSB45 and TSB46, showed the same effect as the natural saponin, initially decreasing protozoa motility but with motility recovering after 24 h (treatment x time interaction, $P<0.05$). Other derivatives, TSB24, TSB47, and TSB52, added at 1 g/L, however, resulted in a greater decrease in protozoa activity over time ($P<0.001$; scores of around 3; no motility or activity evident) with no sign of recovery in motility. Indeed, vacuoles were visible at 24 h suggesting protozoal death (scores of 2.15-2.9). Only few of the hederagenin derivatives (TSB33, TSB34, TSB38 and TSB44) did not show an effect on protozoa motility ($P>0.05$) at any of the concentrations tested. Cholesterol and cholic acid derivatives did not seem to be effective in reducing protozoa motility over time as

246 shown in Figure 4. Only TSB42 when added at 1 g/L showed a slight decrease in protozoa
247 motility after 8 and 24 h of incubation (treatment x time interaction, P=0.017; Figure 4b). A
248 stability index, to estimate the persistence of the saponin effect over time, was calculated as
249 the percentage of the motility at 8 h that remained at 24 h (Figure 5). Whereas the compounds
250 located above the origin on the y-axis were stable (persistent effect on protozoal motility at
251 24 h; e.g. TS24, TSB37, TSB47), those below the origin on the y-axis showed a loss of effect
252 on protozoal motility (recovery of motility after 24 h; e.g. TSB35, TSB46, hederoside B).
253 The derivatives close to or on the origin of the y-axis (e.g. TSB50, TSB51) correspond to
254 those compounds that were less effective against protozoa (scores of about 4.5 at 8 h) but
255 with an effect that was maintained at 24 h.

256 Neither the natural saponin, hederoside B, nor the modified triterpenes or steroids
257 caused a decrease in pH (P>0.05; Table 2); indeed, pH was slightly greater in the presence of
258 TSB35 and TSB37 at 0.5 and 1 g/L (P<0.001) in comparison to the control. Similarly, no
259 effect on the concentration of total VFA was observed in incubations with hederoside B or
260 with most of the derivatives (P>0.05; Table 3). Only TSB35 and TSB36 caused a reduction in
261 the concentration of VFA (P<0.05) when added at 0.5 and 1 g/L. Almost all treatments
262 caused shifts in the molar proportions of VFA towards lower butyrate and higher propionate
263 (P<0.05), to different extents depending on the compound (Tables 5 and 6). Also, some of the
264 derivatives decreased the molar proportion of acetate (Table 4; P<0.05).

265 The natural saponin, hederoside B, decreased acetate and butyrate molar proportions
266 by 8 and 18%, respectively, whereas it increased that of propionate by 35%, in comparison to
267 the control. The greatest effect was observed with TSB35 (hederagenin *bis*-glutarate), TSB37
268 (hederagenin *bis*-betainate dichloride) and TSB47 (hederagenin *bis*-apidate) which, when
269 added at 1 g/L, decreased the molar proportion of acetate and butyrate by 11-13.5% and 35.5-
270 52.7%, respectively, with an increase in propionate of 64.5-84.2%. Cholesteryl succinate

(TSB39) and cholic tri-succinate (TSB41) did not have any effect on the molar proportions of VFA. Cholic succinate (TSB40) caused only a slight decrease in butyrate ($P=0.013$) at 1 g/L, as compared to the control. TSB42 and TSB43 also resulted in decreases in acetate and butyrate and increases in propionate although to a lesser extent than those caused by hederoside B. Molar proportions of branched-chain VFA (BCVFA, i.e. isobutyrate and isovalerate) decreased ($P<0.05$) in incubations with TSB24 (-13%) and TSB38 (-16%) at 1 g/L and TSB50, TSB51, TSB52 and TSB58 at 0.5 and 1 g/L (decreases of 22-24% at 1 g/L; Table 7). TSB43, however, resulted in an increase ($P=0.044$) in BCVFA when added at 1 g/L (+54%; Table 7). This was mainly due to changes in isovalerate rather than isobutyrate (Supplemental Tables S2 and S3).

4. Discussion

The biological activity of saponins depends not only on the type of aglycone but also on the sugar composition and arrangement (Wina et al., 2006). The haemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol with which they form insoluble complexes. It has been shown that monodesmosidic saponins (a single sugar chain) were generally more active than bidesmosidic ones (two sugar chains) (Voutquenne et al., 2002). A further study (Chwalek et al., 2006) testing different hederagenin diglycosides concluded that even the substitution of a monosaccharide with another monosaccharide within the sugar chain may change biological activity of saponins. As far as we know, no studies on the correlation between the haemolytic activity and antiprotozoal activity or on the relationship between saponin structure and antiprotozoal activity in the rumen have been carried out.

295 Although the antiprotozoal effect of saponins has been consistently shown in *in vitro*
296 studies (Wina et al, 2005), it was also found to be transient (Newbold et al., 1997;
297 Teferedegne et al., 1999). This transient nature has been associated to the degradation of
298 saponins, i.e. the cleavage of the glycosidic bonds towards the aglycone leaving the inactive
299 sapogenin behind, by rumen bacteria rather than to the ability of rumen protozoa to become
300 resistant (Newbold et al., 1997). Makkar and Becker (1997) reported the disappearance of
301 saponins from quillaja over time when incubated with buffered rumen fluid, with a reduction
302 of 50% after 12 h and by 100% at 24 h of the incubation. In the present study, we
303 hypothesized that the substitution of the sugar moiety of the saponin with small polar residues
304 would produce sapogen-like analogues that might be resistant to ruminal degradation. Both
305 the acute antiprotozoal activity and the stability of that effect over 24 h of fifteen hederagenin
306 *bis*-esters esterified with two identical groups (Figure 1), and five cholesterol and cholic acid
307 based derivatives carrying one to three succinate residues (Figure 2) was evaluated. Our 5 h
308 *in vitro* incubations results showed that, irrespective of their resistance to degradation, some
309 of the hederagenin derivatives were more effective in reducing protozoa activity than the
310 natural saponin hederoside B. The greatest effect was shown with TSB45, TSB46 and TSB52
311 which reduced protozoa activity by 63-75% when they were incubated at 0.05 g/L.
312 Interestingly among the cholesterol and cholic acid derivatives, TSB39 (cholesteryl
313 succinate) had the lowest antiprotozoal effect and, TSB42 (lithocholic acid succinate) was
314 one of the most effective compounds tested, decreasing protozoa activity by 75% when added
315 at 0.05 g/L. These results agree with the observations of Takechi et al. (1996), who showed
316 that the biological activity that a specific chemical residue may provide is not transferable
317 from one derivative to another. To study if the synthesised derivatives were still effective
318 against protozoa over a longer period of time, *in vitro* incubations were carried out sampling
319 at 0, 4, 8 and 24 h to assess the stability of the derivatives in a mixed rumen population.

Derivatives TSB24, TSB47 and TSB52 seemed to be very effective in causing a decrease in protozoa motility over time without recovery after 24 h, contrary to the results observed for hederoside B and the rest of compounds. Surprisingly, none of the cholesterol and cholic acid derivatives showed an effect on protozoa motility. Although TSB42 had a strong effect in bacterial breakdown by protozoa over 5 h of incubation, little effect on protozoa motility was observed in 24 h *in vitro* batch cultures. These results may suggest a quicker degradation, and thus the loss of activity, of this compound by rumen bacteria as compared with other derivatives tested. It is apparent that the compounds that showed a high level of acute toxicity against protozoa were not always the most stable ones over time. A stability index was calculated as the percentage of the 8 h activity that remained after 24 h (Figure 5). Even though TSB35 reduced protozoa activity by 93% when added at 1 g/L, this compound was among the least stable derivatives. TSB24 and TSB47, however, showed both high toxicity (reduction of protozoa activity of 95-100%) and stability over time.

Most of the hederagenin derivatives did not influence total VFA concentration. However, shifts in the molar proportions of VFA towards lower acetate and butyrate which was compensated by a higher propionate were observed. These changes have been previously reported when using different sources of saponins (Wina et al., 2005; Patra and Saxena, 2009a; Jayanegara et al., 2014). The shifts in the molar proportions of butyrate and propionate shown in the presence of TSB35, TSB37 and TSB47 were, however, much greater than those that would have been expected because of defaunation. A recent meta-analysis showed that defaunation decreased butyrate by 22% with no effect on propionate (Newbold et al., 2015). It should be pointed out that TSB37 was of low purity (66%) and thus, this hederagenin derivative could have been more effective than others with higher purity. However, it is possible that the effects observed in the presence of TSB37 were due to the impurities in this derivative. Although our target in using the synthesised compounds was to

control protozoal activity, other microorganisms may also have been directly or indirectly affected by the derivatives resulting in further effects on rumen fermentation. Indeed, a direct effect of saponins on bacteria, probably mediated by disruption of the cell membrane (Patra and Saxena, 2009a,b; Bodas et al., 2012), has been reported. Similarly, saponins can exert antifungal activity by the interaction with membrane sterols leading to pore formation and loss of membrane integrity (Goel et al., 2008. Patra and Saxena, 2009a,b).

Clearly modifications in the structure of hederagenin resulted in compounds with different biological activities *in vitro*. Whereas some compounds (TSB24) were more effective in reducing protozoa activity and motility, others (TSB37) caused a substantial increase in propionate. If the effect of these compounds can be confirmed *in vivo*, the use of these modified triterpenes in ruminant nutrition will have the potential to improve the efficiency of nitrogen utilization and decrease methane production thus potential boosting productivity.

Conclusion

Most of the hederagenin *bis*-esters, and in particular hederagenin *bis*-sucinate (TSB24), hederagenin *bis*-betainate dichloride (TSB37) and hederagenin *bis*-adipate (TSB47) had a persistent effect against rumen protozoa *in vitro*, shifting the fermentation pattern towards higher propionate and lower butyrate. The confirmation of these effects *in vivo* would help to determine if these novel chemically modified triterpenes could potentially be used in ruminant diets as an effective defaunation agent to, ultimately, increase nitrogen utilization, decrease methane emissions and enhance animal production.

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Author’s contribution

ER, SD, CW, MB, ML, DP, RB and CN contributed to the conception and design of the work; ER and GF conducted the research; ER wrote the manuscript; ER, GF, SD, CW, MB, ML, DP, RB and CN reviewed the manuscript. ER and CN had primary responsibility for the final content. All authors read and approved the final manuscript.

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459 sheep. *Asian-Australas. J. Anim. Sci.* 19, 1580–1587.
460

461 Table 1. Inhibition of protozoa activity (% in respect to the control, no addition) by
462 hederagenin and bile acid derivatives, added at 0.05, 0.1, 0.5 or 1 g/L.
463

	Dose (g/L)			
	0.05	0.1	0.5	1
Hederoside B	5.11	22.0	86.0	84.6
Hederagenin derivatives				
TSB24: Hederagenin bis-sucinate	5.72	18.8	96.5	100
TSB33: Hederagenin bis-(methylethylenglycolacetate)	13.6	29.7	51.3	64.5
TSB34: Hederagenin bis-(MeO-PEG4-carbonate)	7.69	14.1	65.5	69.6
TSB35: Hederagenin bis-glutarate	7.69	36.0	95.5	93.1
TSB36: Hederagenin bis-glycincarbamate	0.55	6.19	55.3	93.8
TSB37: Hederagenin bis-betainate dichloride	16.9	29.1	90.9	94.2
TSB38: Hederagenin bis-sulfate disodium salt	1.32	4.07	47.9	83.9
TSB44: Hederagenin bis-lactate	39.1	86.5	98.3	98.4
TSB45: Hederagenin bis-(2,2-dimethylsuccinate)	63.1	93.6	96.9	97.8
TSB46: Hederagenin bis-(3,3-dimethylglutarate)	75.3	93.0	97.2	96.7
TSB47: Hederagenin bis-adipate	29.6	78.1	98.0	94.0
TSB50: Hederagenin-bis-(diglycolate)	1.06	8.45	75.3	74.3
TSB51: Hederagenin bis-(diglycinate)	1.74	0.29	54.2	63.8
TSB52: Hederagenin bis-(3,3-dimethylsuccinate)	66.7	95.2	98.8	98.4
TSB58: Hederagenin bis-L-tartrate monomethyl ester	0	4.1	95.2	98.2
Cholesterol and Cholic acid derivatives				
TSB39: Cholesteryl succinate	6.42	18.2	17.7	17.6
TSB40: Cholic succinate	25.2	23.5	26.5	42.6
TSB41: Cholic tri-succinate	26.4	21.9	32.9	67.1
TSB42: Lithocholic succinate	75.1	92.8	97.5	97.4
TSB43: Chenodesoxycholic bis-succinate	1.68	5.66	15.5	53.4
SED				
Treatment	4.94***			
Dose	2.16***			
Treatment x Dose	9.88***			

464 SED: Standard error of the difference; ***: P<0.001.
465
466

467 Table 2. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on pH after 24
468 h of incubation (batch culture).
469

	Dose g/L				
	0	0.5	1	SED	P
		pH			
Hederoside B	6.03	-	6.09	0.048	0.253
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	6.16	6.11	6.15	0.039	0.493
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	6.31	6.32	6.31	0.009	0.824
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	6.31	6.31	6.31	0.013	0.924
TSB35: Hederagenin *bis*-glutarate	6.31[a]	6.38[b]	6.38[b]	0.019	0.017
TSB36: Hederagenin *bis*-glycincarbamate	6.31	6.32	6.29	0.019	0.39
TSB37: Hederagenin *bis*-betainate dichloride	6.31[a]	6.36[b]	6.39[c]	0.008	<0.001
TSB38: Hederagenin *bis*-sulfate disodiumsalt	6.41	6.39	6.40	0.014	0.385
TSB44: Hederagenin *bis*-lactate	6.03	6.04	6.04	0.031	0.874
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	6.03[a]	6.12[b]	6.12[b]	0.033	0.051
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	6.16	6.11	6.10	0.034	0.32
TSB47: Hederagenin *bis*-adipate	6.16	6.11	6.15	0.043	0.567
TSB50: Hederagenin *bis*-(diglycolate)	6.16	6.10	6.11	0.036	0.294
TSB51: Hederagenin *bis*-(diglycinate)	6.16	6.06	6.10	0.038	0.122
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	6.16	6.14	6.16	0.042	0.834
TSB58: Hederagenin *bis*-L-tartrate monomethyl ester	6.16	6.12	6.12	0.034	0.464
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	6.18	6.20	6.18	0.031	0.74
TSB40: Cholic succinate	6.03	6.06	6.06	0.026	0.419
TSB41: Cholic tri-succinate	6.03	6.04	6.00	0.019	0.263
TSB42: Lithocholic succinate	6.18	6.19	6.18	0.020	0.876
TSB43: Chenodesoxycholic *bis*-succinate	6.03	6.05	6.03	0.021	0.508

470 [a–c]Means with different superscript differ (n=4).
471
472

473 Table 3. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on total VFA
474 (mM) after 24 h of incubation (batch culture).
475

	Dose g/L				
	0	0.5	1	SED	P
	total VFA (mM)				
Hederoside B	82.5	-	77.2	3.1	0.185
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	70.1	65.4	70.9	4.39	0.448
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	80.1	78.6	76.3	3.88	0.647
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	80.1	75.0	73.9	4.19	0.355
TSB35: Hederagenin *bis*-glutarate	80.1[b]	68.7[a]	66.7[a]	3.14	0.011
TSB36: Hederagenin *bis*-glycincarbamate	80.1[b]	71.2[a]	69.6[a]	3.42	0.045
TSB37: Hederagenin *bis*-betainate dichloride	80.1	70.8	72.1	3.34	0.065
TSB38: Hederagenin *bis*-sulfate disodiumsalt	74.3	70.6	67.3	6.32	0.568
TSB44: Hederagenin *bis*-lactate	82.5	82.0	82.0	2.04	0.958
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	82.5	80.9	73.4	3.47	0.079
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	70.1	73.8	73.8	3.29	0.467
TSB47: Hederagenin *bis*-adipate	70.1	73.1	70.8	3.16	0.620
TSB50: Hederagenin *bis*-(diglycolate)	70.1	73.3	73.3	3.84	0.638
TSB51: Hederagenin *bis*-(diglycinate)	70.1	69.7	74.0	3.84	0.503
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	70.1	69.5	71.3	4.49	0.914
TSB58: Hederagenin *bis*-L-tartrate monomethyl ester	70.1	71.9	72.3	3.64	0.811
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	72.5	72.5	73.3	0.579	0.333
TSB40: Cholic succinate	82.5	80.4	81.3	2.48	0.704
TSB41: Cholic tri-succinate	82.5	81.5	79.1	2.85	0.512
TSB42: Lithocholic succinate	72.5	73.0	66.5	2.46	0.070
TSB43: Chenodesoxycholic *bis*-succinate	82.5	78.9	77.3	2.98	0.279

476 [a–b]Means with different superscript differ (n=4).
477
478

Table 4. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on Acetate (%) after 24 h of incubation (batch culture).

	Dose g/L				
	0	0.5	1	SED	P
	Acetate % of total VFA				
Hederoside B	64.8	-	59.8	0.681	0.005
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	64.1[b]	62.1[b]	59.4[a]	0.837	0.004
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	66.1	65.3	65.2	0.435	0.148
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	66.1	65.4	65.2	0.575	0.31
TSB35: Hederagenin *bis*-glutarate	66.1[c]	60.2[b]	57.2[a]	0.468	<0.001
TSB36: Hederagenin *bis*-glycincarbamate	66.1[b]	64.7[a]	64.1[a]	0.472	0.012
TSB37: Hederagenin *bis*-betainate dichloride	66.1[c]	65.5[b]	58.3[a]	0.751	<0.001
TSB38: Hederagenin *bis*-sulfate disodiumsalt	62.3	62.7	61.8	0.502	0.259
TSB44: Hederagenin *bis*-lactate	64.8	65.4	65.3	0.964	0.787
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	64.8[b]	61.7[a]	61.1[a]	1.177	0.041
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	64.1[b]	61.6[a]	60.3[a]	0.767	0.007
TSB47: Hederagenin *bis*-adipate	64.1[c]	59.1[b]	56.8[a]	0.875	<0.001
TSB50: Hederagenin *bis*-(diglycolate)	64.1	64.4	65.2	1.071	0.582
TSB51: Hederagenin *bis*-(diglycinate)	64.1	65.6	64.7	0.77	0.207
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	64.1[b]	60.8[a]	60.3[a]	0.827	0.008
TSB58: Hederagenin *bis*-L-tartrate monomethyl ester	64.1	65.5	65.3	0.841	0.244
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	61.8	61.8	61.8	0.19	0.993
TSB40: Cholic succinate	64.8	65.0	64.7	0.941	0.948
TSB41: Cholic tri-succinate	64.8	65.5	64.9	1.02	0.744
TSB42: Lithocholic succinate	61.8[b]	61.2[b]	60.1[a]	0.322	0.005
TSB43: Chenodesoxycholic *bis*-succinate	64.8[b]	64.4[b]	61.1[a]	1.25	0.047

[a–c]Means with different superscript differ (n=4).

Table 5. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on Propionate (%) after 24 h of incubation (batch culture).

	Dose g/L				
	0	0.5	1	SED	P
	Propionate % of total VFA				
Hederoside B	20.1	-	27.2	1.04	0.006
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	18.3[a]	25.9[b]	30.5[c]	1.13	<0.001
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	18.6[a]	19.7[ab]	20.3[b]	0.516	0.038
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	18.6[a]	20.1[ab]	20.8[b]	0.715	0.05
TSB35: Hederagenin *bis*-glutarate	18.6[a]	28.0[b]	31.4[c]	0.781	<0.001
TSB36: Hederagenin *bis*-glycincarbamate	18.6[a]	20.7[b]	22.5[c]	0.683	0.004
TSB37: Hederagenin *bis*-betainate dichloride	18.6[a]	24.9[b]	30.6[c]	1.12	<0.001
TSB38: Hederagenin *bis*-sulfate disodiumsalt	20.9[a]	22.3[a]	24.5[b]	0.575	0.002
TSB44: Hederagenin *bis*-lactate	20.1	19.5	20.5	1.10	0.632
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	20.1[a]	27.2[b]	28.3[b]	1.44	0.002
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	18.3[a]	27.4[b]	28.9[b]	0.984	<0.001
TSB47: Hederagenin *bis*-adipate	18.3[a]	30.4[b]	33.7[c]	1.00	<0.001
TSB50: Hederagenin *bis*-(diglycolate)	18.3[a]	20.2[b]	20.5[b]	0.698	0.041
TSB51: Hederagenin *bis*-(diglycinate)	18.3[a]	19.8[b]	22.4[c]	0.496	<0.001
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	18.3[a]	28.6[b]	29.7[b]	1.18	<0.001
TSB58: Hederagenin *bis*-L tartrate monomethyl ester	18.3[a]	19.0[a]	20.9[b]	0.579	0.011
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	21.0	20.8	21.0	0.167	0.458
TSB40: Cholic succinate	20.1	19.7	20.5	1.139	0.817
TSB41: Cholic tri-succinate	20.1	19.1	19.6	0.961	0.643
TSB42: Lithocholic succinate	21.0[a]	22.8[b]	25.0[c]	0.559	0.001
TSB43: Chenodesoxycholic *bis*-succinate	20.1	20.6	23.9	1.476	0.079

[a–c]Means with different superscript differ (n=4).

493 Table 6. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on Butyrate
494 (%) after 24 h of incubation (batch culture).
495

	Dose g/L				
	0	0.5	1	SED	P
	Butyrate % of total VFA				
Hederoside B	12.1	-	9.83	0.427	0.013
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	14.3	8.8	7.2	0.606	<0.001
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	11.7	11.5	11.1	0.25	0.1
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	11.7^{b}	11.2^{ab}	10.7^{a}	0.231	0.017
TSB35: Hederagenin *bis*-glutarate	11.7^{b}	7.92^{a}	7.54^{a}	0.253	<0.001
TSB36: Hederagenin *bis*-glycincarbamate	11.7^{c}	11.2^{b}	10.1^{a}	0.150	<0.001
TSB37: Hederagenin *bis*-betainate dichloride	11.7^{c}	9.17^{b}	7.70^{a}	0.375	<0.001
TSB38: Hederagenin *bis*-sulfate disodiumsalt	12.8^{c}	11.3^{b}	10.2^{a}	0.4	0.002
TSB44: Hederagenin *bis*-lactate	12.1^{b}	11.7^{b}	11.0^{a}	0.173	0.003
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	12.1^{b}	7.74^{a}	7.62^{a}	0.394	<0.001
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	14.3^{b}	8.26^{a}	7.76^{a}	0.571	<0.001
TSB47: Hederagenin *bis*-adipate	14.3^{b}	7.35^{a}	6.78^{a}	0.608	<0.001
TSB50: Hederagenin *bis*-(diglycolate)	14.3^{b}	12.6^{a}	11.7^{a}	0.506	0.005
TSB51: Hederagenin *bis*-(diglycinate)	14.3^{b}	11.7^{a}	10.2^{a}	0.606	0.001
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	14.3^{b}	7.86^{a}	7.33^{a}	0.746	0.001
TSB58: Hederagenin *bis*-L-tartrate monomethyl ester	14.3^{c}	12.5^{b}	11.2^{a}	0.383	<0.001
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	12.9	13.0	12.8	0.094	0.341
TSB40: Cholic succinate	12.1^{b}	11.8^{b}	11.3^{a}	0.178	0.013
TSB41: Cholic tri-succinate	12.1	12.0	12.1	0.322	0.938
TSB42: Lithocholic succinate	12.9^{c}	11.4^{b}	10.2^{a}	0.257	<0.001
TSB43: Chenodesoxycholic *bis*-succinate	12.1^{c}	11.5^{b}	10.9^{a}	0.176	0.002

496 $^{a–c}$Means with different superscript differ (n=4).
497
498

499 Table 7. Effect of Hederagenin and bile acid derivatives, added at 0.5 or 1 g/L, on branched
500 chain volatile fatty acids (BCVFA) (%) after 24 h of incubation (batch culture).
501

	Dose g/L				
	0	0.5	1	SED	P
	BCVFA % of total VFA				
Hederoside B	1.95	-	2.08	0.161	0.474
Hederagenin derivatives					
TSB24: Hederagenin *bis*-sucinate	2.08^{b}	1.96^{ab}	1.81^{a}	0.081	0.045
TSB33: Hederagenin *bis*-(methylethylenglycolacetate)	2.47	2.29	2.29	0.126	0.307
TSB34: Hederagenin *bis*-(MeO-PEG4-carbonate)	2.47^{a}	2.19^{ab}	2.11^{b}	0.124	0.056
TSB35: Hederagenin *bis*-glutarate	2.47	2.23	2.65	0.216	0.219
TSB36: Hederagenin *bis*-glycincarbamate	2.47	2.33	2.34	0.229	0.775
TSB37: Hederagenin *bis*-betainate dichloride	2.47	2.26	2.35	0.082	0.103
TSB38: Hederagenin *bis*-sulfate disodiumsalt	2.64^{b}	2.42^{ab}	2.22^{a}	0.120	0.032
TSB44: Hederagenin *bis*-lactate	1.95	2.30	2.07	0.275	0.469
TSB45: Hederagenin *bis*-(2,2-dimethylsuccinate)	1.95	2.36	1.85	0.321	0.305
TSB46: Hederagenin *bis*-(3,3-dimethylglutarate)	2.08^{b}	1.68^{a}	2.01^{b}	0.096	0.012
TSB47: Hederagenin *bis*-adipate	2.08	1.91	1.77	0.166	0.263
TSB50: Hederagenin *bis*-(diglycolate)	2.08^{b}	1.79^{a}	1.62^{a}	0.11	0.016
TSB51: Hederagenin *bis*-(diglycinate)	2.08^{b}	1.69^{a}	1.63^{a}	0.08	0.003
TSB52: Hederagenin *bis*-(3,3-dimethylsuccinate)	2.08^{b}	1.59^{a}	1.58^{a}	0.088	0.002
TSB58: Hederagenin *bis*-L-tartrate monomethyl ester	2.08^{b}	1.86^{b}	1.57^{a}	0.091	0.004
Cholesterol and Cholic acid derivatives					
TSB39: Cholesteryl succinate	3.10	3.23	3.13	0.261	0.879
TSB40: Cholic succinate	1.95	2.40	2.45	0.209	0.1
TSB41: Cholic tri-succinate	1.95	2.30	2.31	0.1772	0.141
TSB42: Lithocholic succinate	3.10	3.63	3.49	0.191	0.203
TSB43: Chenodesoxycholic *bis*-succinate	1.95^{a}	2.52^{ab}	3.00^{b}	0.319	0.044

502 $^{a–b}$Means with different superscript differ (n=4).
503
504

505 **Figure legends**

506

507 Figure 1. Structure of Hederagenin derivatives.

508 Figure 2. Structure of Cholesterol and Cholic acid derivatives and Hederoside B.

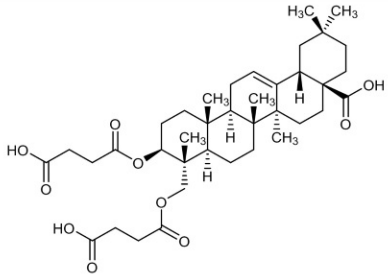
509 Figure 3. Protozoa motility over 24 h in the absence (control) or presence of different
510 hederagenin derivatives at 0.5 (**A**) and 1 g/L (**B**). Hederoside B was used as a positive control
511 at 1 g/L. Error bars indicate the standard error of the difference for each time point (n = 4).

512 Figure 4. Protozoa motility over 24 h in the absence (control) or presence of different
513 cholesterol and cholic acid derivatives at 0.5 (**A**) and 1 g/L (**B**). Hederoside B was used as a
514 positive control at 1 g/L. Error bars indicate the standard error of the difference for each time
515 point (n = 4).

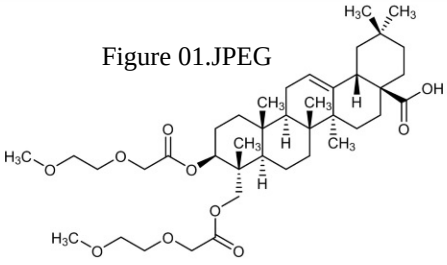
516 Figure 5. Stability index (calculated as the percentage of the motility at 8 h that remained at
517 24 h) against motility scores at 8 h in the presence of hederagenin and cholesterol and cholic
518 acid derivatives and hederoside B (HB) at 1 g/L. Error bars indicate the standard error of the
519 difference (n = 4).

520

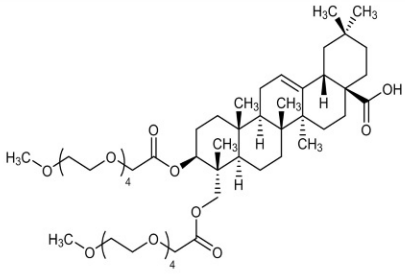
Figure 01.JPEG



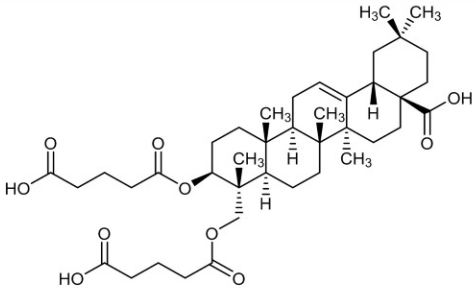
Hederagenin *bis*-succinate (TSB24)



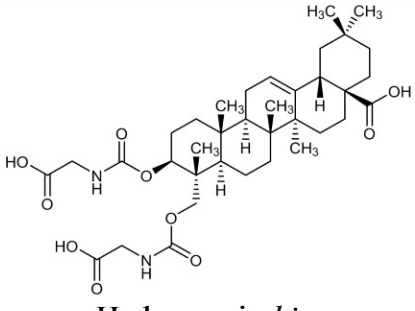
Hederagenin *bis*-(methylethylenglycolacetate) (TSB33)



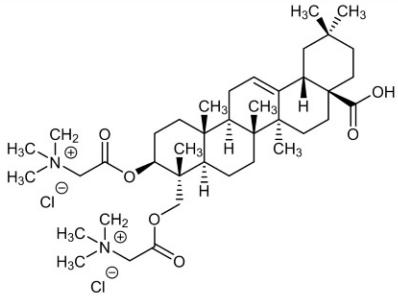
Hederagenin *bis*-(MeO-PEG4-carbonate) (TSB34)



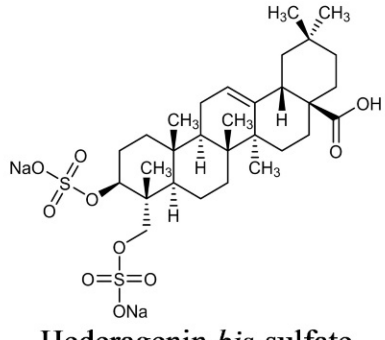
Hederagenin *bis*-glutarate (TSB35)



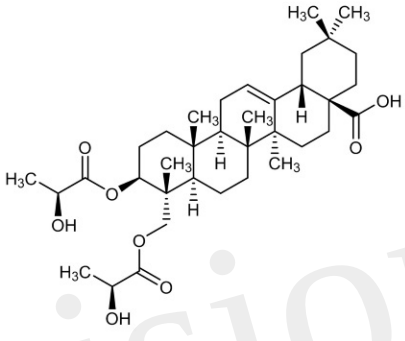
Hederagenin *bis*-glycincarbamate (TSB36)



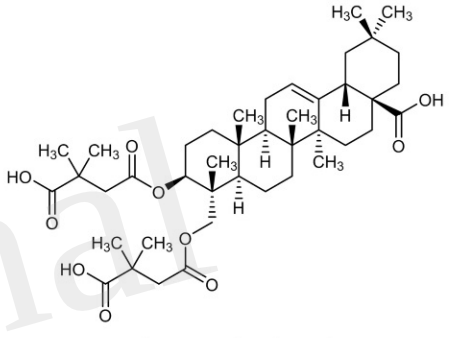
Hederagenin *bis*-betainate dichloride (TSB37)



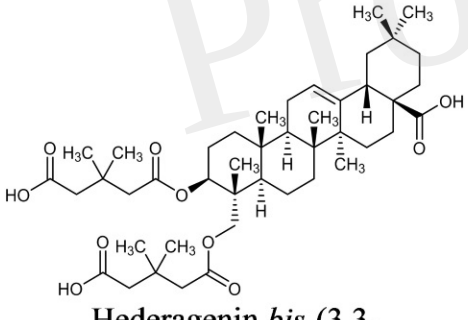
Hederagenin *bis*-sulfate disodiumsalt (TSB38)



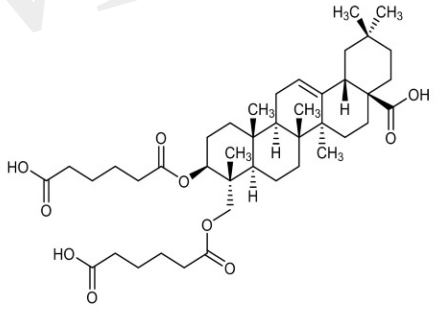
Hederagenin *bis*-lactate (TSB44)



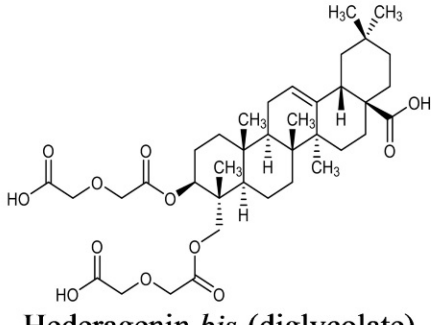
Hederagenin *bis*-(2,2-dimethylsuccinate) (TSB45)



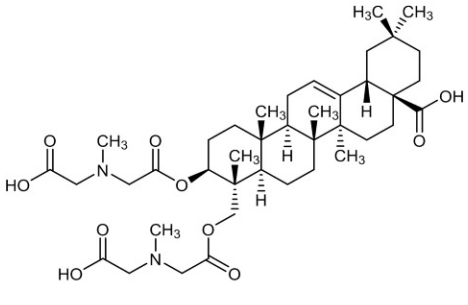
Hederagenin *bis*-(3,3-dimethylglutarate) (TSB46)



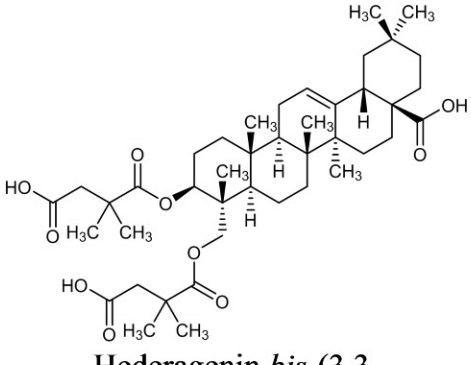
Hederagenin *bis*-adipate (TSB47)



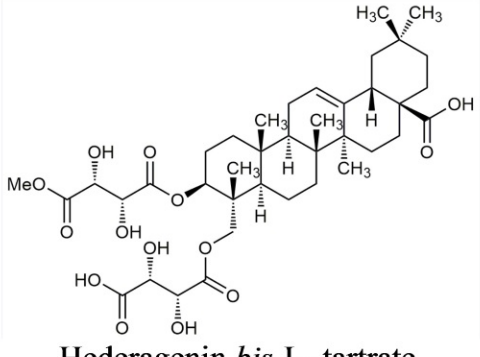
Hederagenin *bis*-(diglycolate) (TSB50)



Hederagenin *bis*-(diglycinate) (TSB51)

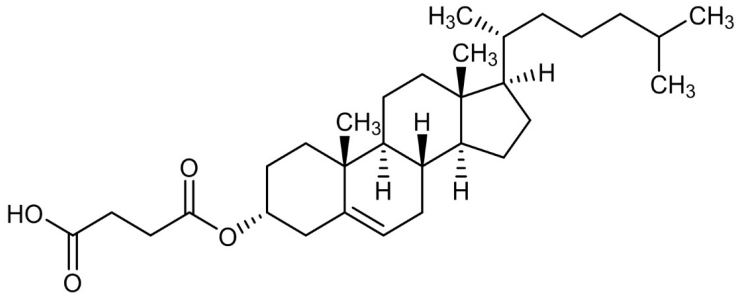


Hederagenin *bis*-(3,3-dimethylsuccinate) (TSB52)

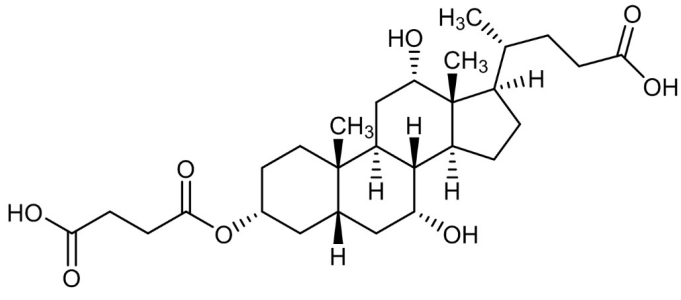


Hederagenin *bis*-L- tartrate monomethyl ester (TSB58)

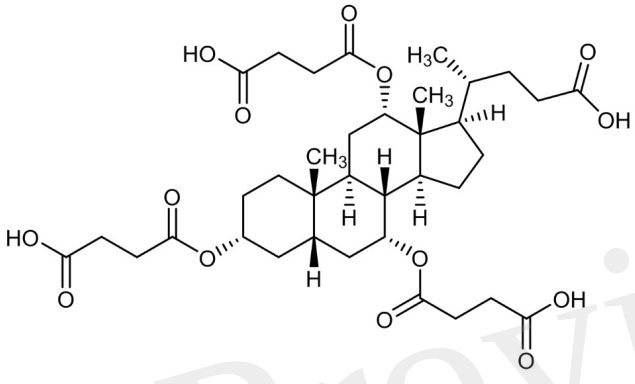
Figure 02.JPEG



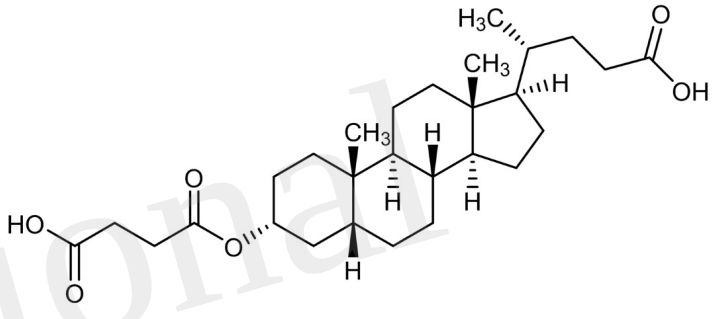
Cholesteryl succinate (TSB39)



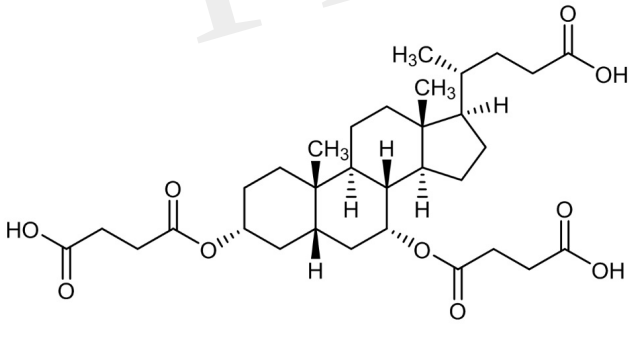
Cholic succinate (TSB40)



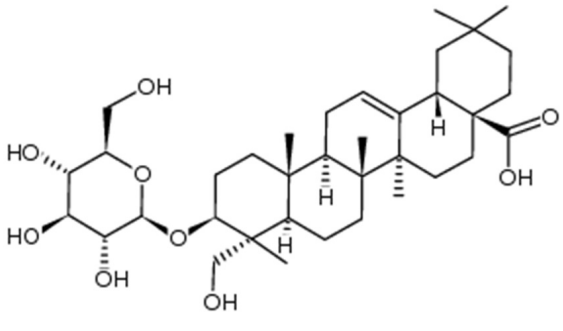
Cholic tri-succinate (TSB41)



Lithocholic succinate (TSB42)

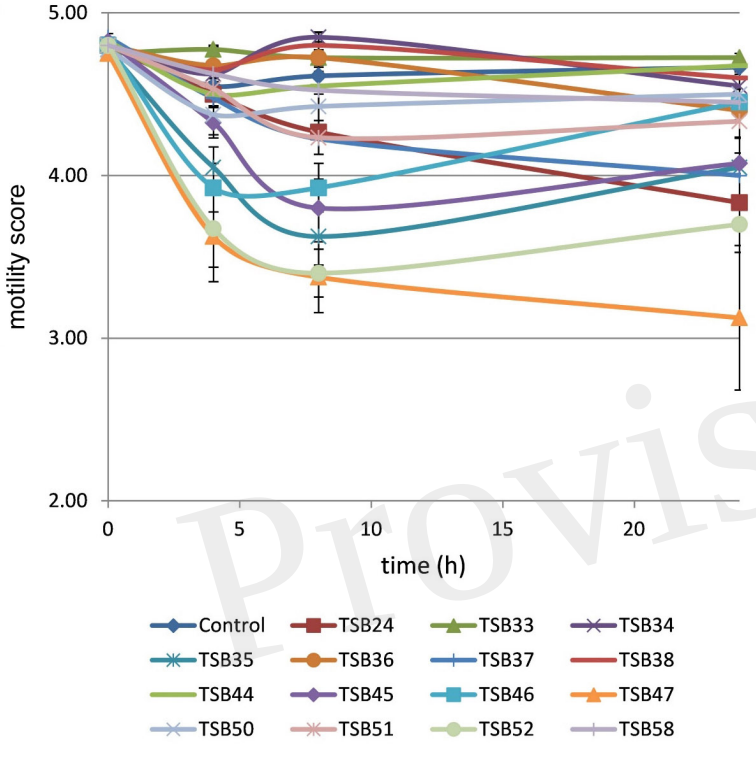


Chenodesoxycholic *bis*-succinate (TSB43)



Hederoside B

Figure 03.JPEG



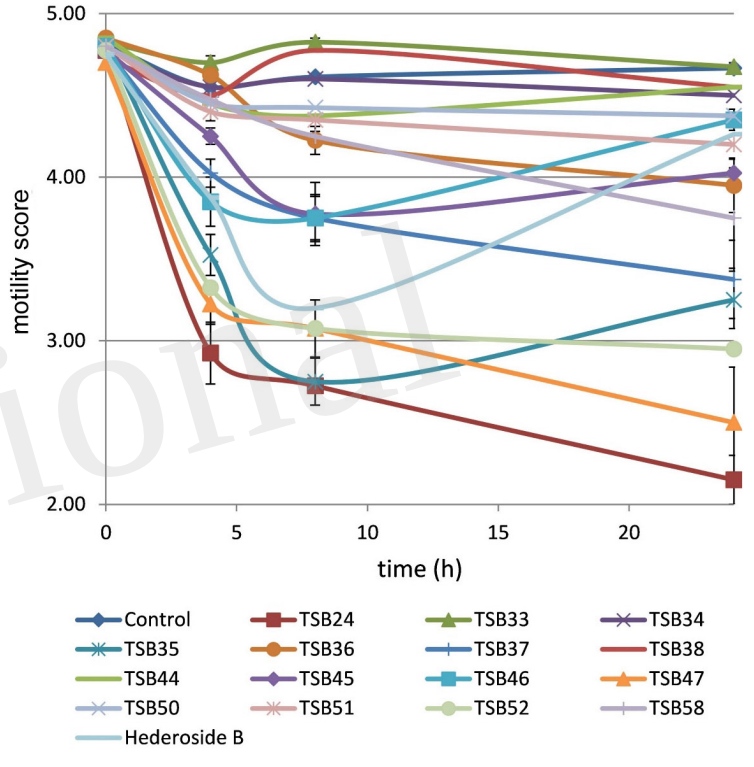
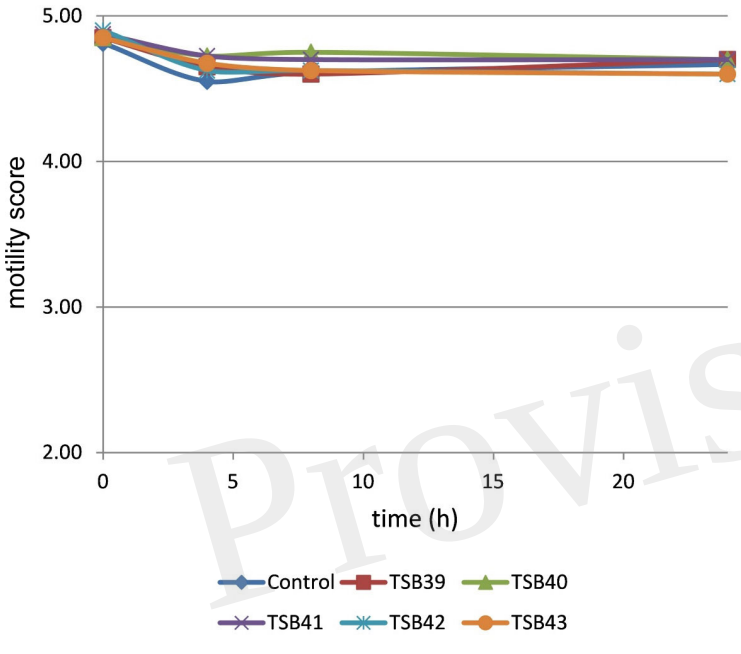


Figure 04.JPEG



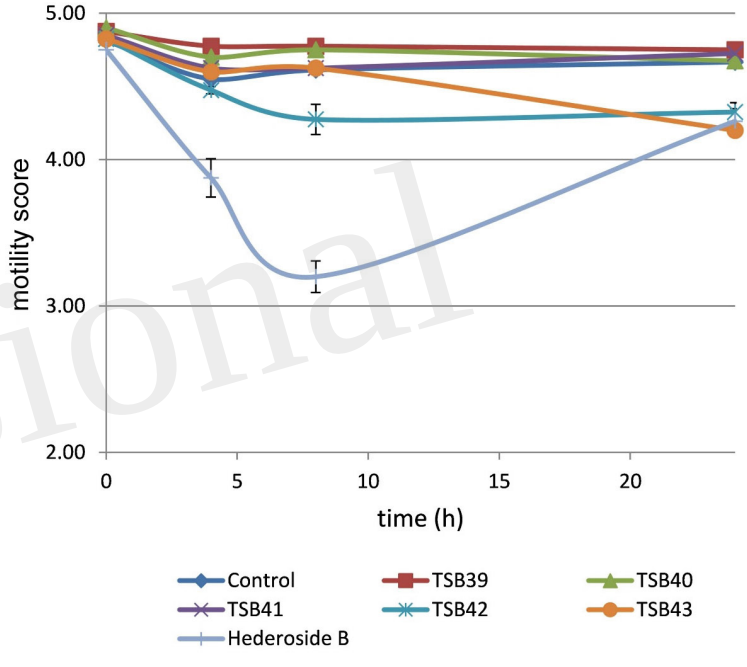


Figure 05.JPEG