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Ellagitannins with a Glucopyranose Core Have Higher Affinity to Proteins than Acyclic Ellagitannins by Isothermal Titration Calorimetry

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1   **ABSTRACT:**

2   The thermodynamics of the interactions of different ellagitannins with two proteins, namely bovine serum

3   albumin (BSA) and gelatin, were studied by isothermal titration calorimetry. Twelve individual ellagitannins,

4   including different monomers, dimers and a trimer, were used. The studies showed that several structural

5   features affected the interaction between the ellagitannin and the protein. The interactions of ellagitannins with

6   proteins were stronger with gelatin than with BSA. The ellagitannin-gelatin interactions contained both the

7   primary stronger and the secondary weaker binding sites. The ellagitannin-BSA interactions showed very weak

8   secondary interactions. The ellagitannins with a glucopyranose core had stronger interaction than C-glycosidic

9   ellagitannins with both proteins. In addition, the observed enthalpy change increased as the degree of

10   oligomerization increased. The stronger interactions were also observed with free galloyl groups in the

11   ellagitannin structure and with higher molecular flexibility. Other smaller structural features did not show any

12   overall trend.

13

14   **KEYWORDS:** *binding, bovine serum albumin, ellagitannin, gelatin, isothermal titration calorimetry,*

15   *thermodynamics*

17   **INTRODUCTION**

18

19   Tannins are plant secondary metabolites, which could also be called plant specialized metabolites[1,2]. Plants

20   produce them in their tissues to protect themselves against, for example pathogens and insect herbivores.

21   Tannins are polyphenols that have the ability to bind and precipitate proteins and they can be divided into three

22   groups: hydrolysable tannins, proanthocyanidins (syn. condensed tannins) and phlorotannins. Hydrolyzable

23   tannins are further divided into simple gallic acid derivatives, gallotannins and ellagitannins. Ellagitannins

24   (ETs) are a structurally complex group and individual structures vary from simple hexahydroxydiphenoyl

25   (HHDP) esters to high oligomers with both varying degree of oligomerization and types of bonds between the

26   monomers[3,4] ETs have been stated as the most promising tannin class with potent biological activities, such

27   as antimicrobial, antioxidant and antiparasitic activities[3,5–8]

28   Some dietary tannins can have several beneficial effects in animal nutrition and health, for

29   example, through enabling a better utilization of feed proteins, generating anthelmintic effects against

30   gastrointestinal nematodes, and lowering nitrogenous and methane emissions[8–13] The interactions between

31   tannins and proteins plays an important role in these bioactivities observed. Tannins may bind dietary proteins

32   and thus can reduce the degradation of these proteins in the rumen and they may also enhance the amount of

33   protein available for digestion in the small intestine[12] Tannins can also interact with digestive enzymes, such

34   as α-glycosidase, α-amylase, lipase, pepsin, trypsin, and chymotrypsin, and thus inhibit their enzymatic

35   activities[14,15] These interactions are mostly regulated by non-covalent binding, i.e. van der Waals forces,

36   hydrogen bonding, and other electrostatic forces[14] Tannins can form soluble and/or insoluble complexes with

37   proteins, and the tannin−protein interactions are both tannin- and protein-specific[16] The studies on the effects

38   of 27 individual ETs and 7 galloylglucoses and gallotannins on the egg hatching of pathogenic parasite

39   *Haemonchus contortus* showed that several compounds have antiparasitic properties and clear structure-

40   activity relationships were observed[8] The mechanisms of action remained unclear but the main reason seemed

41   to be that tannins bind to egg shell proteins and thereby disturb the egg hatching process[8]

42   Isothermal titration calorimetry (ITC) is an ideal technique to measure biological binding

43   interactions, such as the interactions between the tannin and the protein[17–24] ITC can be used to measure the

44   thermodynamics of the interaction, i.e. the binding constant K, the enthalpy of binding (ΔH_{obs}) and the

45   stoichiometry or number of binding sites (n).[24] Most of the ITC studies on the interaction between tannins and

46   proteins have focused on proanthocyanidins.[19–21,23] Oligomeric and polymeric proanthocyanidins cannot be

47   purified as individual compounds and therefore mainly proanthocyanidin fractions have been used. This makes

48   the interpretation of results and the determination of thermodynamic binding parameters more difficult.[23] The

49   only exceptions are monomeric flavan-3-ols and cocoa proanthocyanidins consisting of epicatechin monomers

50   which have been separated into different oligomers and studied in detail by ITC.[20,23]

51   In our previous study, we utilized a unique series of oligomeric tellimagrandin I –based ETs

52   and studied their interaction with BSA by ITC.[22] The ET series from tetramers to octa−undecamers enabled

53   the evaluation of the effect of the molecular size on the interaction and we could decouple the other structural

54   features. The interactions of ETs with BSA revealed strong similarities: Enthalpy showed an increasing trend

55   from the dimer to larger oligomers. Our studies highlighted the importance of molecular flexibility to maximize

56   binding between the tannin and protein surface.[22] In this study, ET structures were selected so that they differed

57   in the molecular flexibility and size and that they had different structural features (Fig. 1). These features

58   included, for example, the tautomeric forms of the glucose core (glucopyranose versus acyclic core), different

59   functional groups in their structures or the position of free hydroxyl group at C-1 (α and β anomers). In

60   addition, two proteins were used: BSA, a model for the globular proteins, and gelatin, a model for flexible

61   proline-rich proteins.

62   Altogether, 12 ETs were selected and purified from different plant sources and their interactions

63   were studied with BSA and gelatin by ITC. The aim was to broaden the knowledge on tannin-protein

64   interactions and to study in detail how efficient different ETs are at binding with different proteins and to

65   characterize the thermodynamics of these bindings.

67　　**MATERIALS AND METHODS**

68

69　　　　　**Isolation and Characterization of ETs.** ETs were extracted, isolated and purified from plant

70　　extracts and characterized by the methods previously described.[4,22,25–28] The plant material was collected and

71　　placed directly into 10 bottles of 1 L, which were then immediately filled with acetone, transferred to the

72　　laboratory, and stored in a cold room (4 °C) prior to the isolation of ellagitannins.[22] The preliminary

73　　fractionation was performed by Sephadex LH-20 chromatography and the final purifications of ellagitannins

74　　were made by preparative and semipreparative HPLC; all steps were followed by UPLC-ESI-MS. [22] The

75　　ellagitannins were identified based on their chromatographic elution order, UV spectra, molecular ions and

76　　characteristic fragment ions based on our previous work and literature as shown in Table 1. Pure ellagitannins

77　　were concentrated to the water phase and freeze-dried. The individual ellagitannins, their purities obtained by

78　　UPLC at 280 nm and the electrospray ionization mass spectrometric identification are presented in Table 1.

79　　.Monomeric ETs with a glycopyranose core, tellimagrandin I and tellimagrandin II (1 and 2 in Fig. 1), were

80　　isolated from the meadowsweet inflorescence (*Filipendula ulmaria*)[29–31] and geraniin (3 in Fig. 1) from the

81　　wood cranesbill leaves (*Geranium sylvaticum*)[32]. Acyclic ETs castalagin and vescalagin (4 and 5 in Fig. 1)

82　　were isolated from the purple loosestrife flowers and leaves (*Lythrum salicaria*)[30,31,33]) and castavaloninic and

83　　vescavaloninic acids (6 and 7 in Fig. 1) from the English oak acorns (*Quercus robur*)[34,35].The stereochemistry

84　　of castalagin and vescalagin were lately reinvestigated by computational methods and the

85　　nonahydroxytriphenoyl group (NHTP) was found to exist in (S,R) configuration.[36,37] Therefore, it is feasible

86　　that the NHTP group of vescavaloninic and castavaloninic acids is also in (S,R) configuration.　Dimeric

87　　agrimoniin (8 in Fig. 1) was from the silverweed leaves (*Potentilla anserina*)[25,31,38], gemin A (9 in Fig. 1) from

88　　the wood avens leaves (*Geum urbanum*)[25,39], and sanguiin H-6 and roshenin C (10 and 11 in Fig. 1) from the

89　　raspberry leaves (*Rubus idaeus*)[25,38,40]. In addition, trimeric lambertianin C (12 in Fig. 1) was isolated from the

90　　raspberry leaves.

91　　　　　**Isothermal Titration Calorimetry.** A NanoITC instrument (TA Instruments Ltd., Crawley,

92　　West Sussex, UK) was used to measure the thermodynamics of titrations of ET into BSA (purity $\geq$ 98%,

93　　lyophilized powder, 66 kDa; Sigma-Aldrich, St. Louis, US, CAS 9048-46-8) or into gelatin (Gelatin, type B

94   derived from lime-cured tissue, purity approx. 225 Bloom which is proportional to the average molecular mass

95   of 50 kDa, Sigma-Aldrich, CAS 9000-70-8). The measurements were performed as earlier described.[22] All

96   solutions were prepared in 50 mM citrate buffer adjusted to pH 6. In a typical measurement for the interaction

97   between the ET and BSA, 20 or 30 μM BSA solution was placed in the 950 μL sample cell of the calorimeter

98   and 3 mM ET solution was loaded into the injection syringe. The ET studied was titrated into the sample cell

99   at 298 K as a sequence of 24 injections of 10 μL aliquots. The time delay between the injections was 360 s.

100   Each ET-BSA interaction was measured at least with three replicates.

101   In a typical measurement for the interaction between ET and gelatin, different gelatin

102   concentrations were used depending on the strength of the interactions. The different gelatin contents were

103   0.3, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/mL. To get the molarities of the solution, the content of the solution was

104   divided with the estimated molecular mass of gelatin (50 000 g/mol). The molarities of the gelatin solutions

105   were thus approximately 6, 10, 15, 20, 30 and 40 μM, respectively. Each ET-gelatin interaction was measured

106   at least with three replicates.

107   Raw data were obtained as plots of heat (μJ) against injection number and exhibited a series of

108   peaks for each injection. Examples of thermograms are available in figures S1-S6 in Supporting Information.

109   The plots of raw heat data were transformed using the NanoAnalyze Data Analysis software (version 2.4.1.,

110   TA instruments) to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ/mol) against

111   molar ET:BSA ratio. The control data of ellagitannin titrated into buffer were always subtracted from the

112   sample data as it was known that ellagitannins tend to selfassociate into aggregates and then undergo an

113   endothermic process of deaggregation when titrated from the syringe into buffer.[19,22] Data fits and estimated

114   binding parameters were obtained in two different ways: using a single set of multiple binding sites and a

115   model for two independent sets of multiple binding sites. The quality of fits was determined by standard

116   deviation.

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118

120   **RESULTS AND DISCUSSION**

121

122   **ITC Binding Isotherms and Data Fitting for the ET/BSA Interaction**. The interactions of twelve individual

123   ETs (Figure 1) with BSA were studied by ITC. These ETs included three cyclic ET monomers, namely

124   tellimagrandin I and tellimagrandin II and geraniin (ITC isotherms shown in Figure 2), four ET dimers, i.e.

125   sanguiin H-6, roshenin C, agrimoniin and gemin A, and one ET trimer, i.e. lambertianin C (Fig. 3), and four

126   acyclic ET monomers, namely castalagin, vescalagin, castavaloninic acid and vescavaloninic acid (Fig. 4).

127   Figures 2 and 3 show the ITC binding isotherms for the cyclic ET monomers, dimers and trimer

128   binding to BSA. For these ET-BSA systems, an exothermic interaction was observed, and the interaction

129   became less exothermic with the increasing injection number (increase in the tannin to protein molar ratio) as

130   the binding sites of BSA became saturated. All experiments were performed in triplicate and using different

131   concentrations of BSA, varying from 10 µM to 40 µM depending on the ET and the observed changes of

132   enthalpy were detected. The interaction was not affected by BSA concentration, which suggested no evidence

133   of co-operative binding as previously reported for oligomeric ETs.[22]

134   The data fittings were performed using two different binding models. One assumed a single set

135   of multiple binding sites (later called as single-site model) and the other one two independent sets of multiple

136   binding sites (later called as two-site model).[21] For sanguiin H-6 and lambertianin C, the first data points of

137   the titration isotherm were not used in order to fit the data both in two-site and one-site models (Fig. 3C and

138   D). We have observed similar shapes in tannin-protein binding isotherms before and have suggested that this

139   could indicate synergism in protein binding, such that the presence of ellagitannin already bound to gelatin has

140   an effect on the binding of subsequent tannin molecules.[19] There are also other possibilities that could explain

141   this trend since there could be competing endothermic and exothermic interactions at play. In general, it can

142   be seen that both binding models visually fit the data well for all of the ET/BSA systems with little difference

143   in the agreement of the data fit curves and the data points for both binding models. Where a two-site model

144   was used the second site showed usually a weaker binding with K_{a2} in the region of 10^1-10^3 M^{-1} (Table 2 and

145   3) and a single-site model was able to equally well represent the data. In some cases, see for example

146   lambertianin C and BSA (Fig. 3D), the two-site model clearly exhibited a slightly better fit to the data than the

147   single-site binding model. Similar observation were made in our previous study[22], and therefore, the fit

148   parameters for both binding models are shown in Tables 2 and 3. In addition, the estimated entropies for the

149   ellagitannin-BSA interactions are presented as Supporting Information in Table S1. However, as all the

150   interactions between ETs and BSA fit to the one-site model there is no clear justification for increasing the

151   complexity of the model used to fit this data and therefore we will discuss the interactions between BSA and

152   ETs based on the fittings obtained by the single-site model.

153   The ETs could be classified into three different groups based on the strength of their interactions

154   with BSA; i.e. stronger, moderate and weaker interaction: i) Six ETs with a glupyranose core, i.e.

155   tellimagrandin I, tellimagrandin II, agrimoniin, gemin A, sanguiin H-6, and lambertianin C, had the strongest

156   interactions with BSA and the thermodynamic binding parameters could be estimated for these interactions

157   (Tables 2 and 3). ii) Two ETs with a glucopyranose core, i.e. geraniin and roshenin C, had moderate

158   interactions with BSA (Figs. 2 and 3), and for these it was difficult to produce a clear fit to the data and

159   therefore we have less confidence in our estimated thermodynamic binding parameters for these ET/BSA

160   interactions. iii) All acyclic ETs, i.e. castalagin, vescalagin, castavaloninic acid, and vescavaloninic acid, had

161   no interaction or very weak interactions with BSA (Fig. 4) and no fits or thermodynamic binding parameters

162   were obtained. Therefore, it was immediately evident that binding of the ETs to BSA were stronger for ETs

163   with a cyclic core than for those with an acyclic glucose core.

164   Both sanguiin H-6 and lambertianin C binding to BSA exhibited an increase in ΔH during early

165   injection numbers (low molar ratio) which indicates that the presence of ET in the BSA/ET solution results in

166   an initial increase in binding. This may indicate that previously bound ET on the protein is able to facilitate

167   the binding of subsequent ET molecules injected into the solution. Such features in ITC binding isotherms

168   have been seen in other studies as an indication of this kind of co-operative binding. We also noted above that

169   gemin A exhibited different behavior in cases where the experimental procedure was different, and this may

170   be explained if the method was able to provide enough time between injections to maximize the effect of this

171   co-operative binding.

172   Table 2 and Figure 2 compare our three monomeric cyclic ETs and show that the binding with

173   BSA is strongest for tellimagrandin I ($K_a = 1.8 \times 10^4 \, \text{M}^{-1}$) compared to tellimagrandin II ($K_a = 7.3 \times 10^{-3} \, \text{M}^{-1}$).

174   As could be expected, the weakest binding with BSA of our ET monomers studied was with geraniin, which

175   also exhibits a more rigid constrained structure compared to tellimagrandin I and II. For all these three ETs,

176   ΔH is similar. It is not clear if there is any substantial trend or information that can be gained from the values

177   of n (number of ET molecules binding to each BSA molecule). Our fit consistently gave tellimagrandin II a

178   relatively high value for n compared to the other ETs (Table 2). Previously, there has been a suggestion that

179   weak binding between tannins and BSA can result from unspecific adsorption that has a weak binding affinity

180   to the BSA surface. The weak K_a seen for tellimagrandiin II coincides with a higher fitted value for n and may

181   well indicate such an adsorption event. To further support the link between high values for n and non-selective

182   adsorption, tellimagrandin II has poorer water-solubility than other ETs (based on unpublished octanol-water

183   coefficients), and thus a greater tendency towards surface adsorption. This type of adsorption might also be

184   expected for ETs with greater flexibility in their structure allowing for less conformational restraints and

185   increased ability to non-selectively binding to protein surfaces. [17,18] Our dimeric and trimeric ETs show

186   variations in binding that are greater than that seen for tellimagrandin II and geraniin, with the trimeric

187   lambertianin C exhibiting the strongest interaction ($K_a = 1.1 \times 10^5$ M^{-1}). Overall, we see a link between the

188   oligomerization and the strength of interaction between ET and BSA. As seen here, our previous studies found

189   that the interaction of monomeric tellimagrandin I with BSA was stronger, in terms of K_a, than expected in

190   relation to the oligomeric series of ETs.[22] However, that oligomeric series contained similar monomeric units

191   in all the oligomers, which enabled the direct comparison of the interaction between the different oligomers

192   based on the number of monomeric units and without the effects of the other structural features, such as

193   functional groups.[22] In general for polyphenols, the increase in the binding affinity with the molecular size

194   have also been observed previously even if other differences in the molecular structures are present.[41]

195   However, for quercetin and quercetin 3-O-β-D-glucopyranoside binding with BSA, the opposite has been

196   reported.[42]

197   The dimeric ETs that we investigated exhibited similar behavior in terms of their binding to

198   BSA, agrimoniin, gemin A and sanguiin H-6 had equilibrium binding constants varying between 1.1-1.7×10^4

199   M^{-1}, and similar values for ΔH and n. Agrimoniin and gemin A are structurally closely similar; the main

200   difference is that agrimoniin contains four HHDP groups whereas gemin A has three HHDP groups and two

201   free galloyl groups and that the orientation at C-1 of the glucose is α in agrimoniin and β in gemin A. Two of

202   the binding parameters for gemin A, $\Delta H_1 = -45$ kJ mol^{-1} and n = 14, are similar to the previously measured

203   values, $\Delta H_1 = -47$ kJ mol^{-1} and $n_1 = 13$, but the equilibrium binding constant 1.1×10^4 M^{-1} is different to the

204   previous one 1.8×10^6 M^{-1}.[17] In previous study, ETs were titrated into the sample cell in two titration events

205   where the syringe was filled within the run, i.e. first the sequence of 24 injections, then the filling of the syringe

206   and then the sequence of 24 injections.[17] The current measurements were performed as a single titration event

207   without any additional distractions to the titration, and this means that the titration volume and time taken for

208   the experiment to complete are different. Sanguiin H-6 and roshenin C only differ by the latter lacking one

209   HHDP group, but still they showed very different behaviors in terms of BSA binding. Our fits for roshenin C

210   are poor because of the observed weakness of the interaction with small variation in ΔH and show significant

211   variability in terms of the strong binding site between the two binding model fits. We are unable to identify

212   any structural reason to explain why the roshenin/BSA interaction is weak and we are unable to provide

213   confident fit parameters for this ET. Nevertheless, this finding shows that not only galloyls are important for

214   increasing tannin-protein interaction, but also the presence vs. absence makes a difference.

215   Acyclic ETs castalagin, vescalagin, castavaloninic and vescavaloninic acids had very weak

216   interactions with BSA based on the isotherms (Fig. 4). No reliable fits or thermodynamic binding parameters

217   were obtained. The weak interaction cannot be due to low water-solubility as acyclic ETs are very water-

218   soluble.[28] The other reason could be the relatively rigid conformation of acyclic ETs having NHTP groups in

219   their structures (Fig. 1). The interaction between vescalagin and BSA has been previously studied and found

220   to be weak in comparison to other ETs.[17] The interaction between acyclic ETs and BSA were so weak that we

221   could not evaluate the effects of other structural features, such as the role of free COOH present in

222   vescavaloninic and castavaloninic acids or the effects of the α or β orientation at C-1 of the glucose.

223   **ITC Binding Isotherms and Data Fitting for the ET/Gelatin Interaction**. The interaction of

224   the ETs with gelatin is shown in Figures 5-7 and Tables 4-5. The cyclic ET monomers in Fig. 5 and the cyclic

225   ET dimers and trimer in Fig. 6 all show an exothermic interaction between the ET and gelatin showing a

226   gradual decrease in exothermicity as the binding sites of gelatin become saturated. In general, the interaction

227   was stronger between gelatin and ETs than between BSA and ETs. As for our ET-BSA data, the data fittings

228   were performed using the single-site model and the two-site model. However, for the interaction with gelatin

229   it can be seen that overall the two-site model fit the data better. For example, for the interaction between gemin

230   A and gelatin, the two-site model clearly visually exhibited a closer fit (Fig. 6B) and the calculated SD for the

231   fits and thermodynamic binding parameters (Table 5) also supported the presence of the secondary binding

232   site. Similar observations were made for all the other ETs (Figs. 5 and 6, Tables 4 and 5). For the trimer ET,

233   lambertianin C (Fig. 6D), the data showed no evidence of a second-site binding and the ET-gelatin binding

234   isotherm showed a strong primary binding site. As for our BSA data, the fit parameters for both binding models

235   are shown in Tables 4 and 5. In addition, the estimated entropies for the ellagitannin-gelatin interactions are

236   presented as Supporting Information in Table S1. Given that the interaction between gelatin and ETs are

237   approximately ten-fold stronger (see K_{a1} values), it may not be surprising that a second, perhaps less specific

238   binding (or non-selective adsorption to the protein) could be observed.

239   Similarly to the interaction between different ETs and BSA, it was evident that the interactions

240   between ETs and gelatin were stronger for ETs with a glucopyranose core than for acyclic ETs as the observed

241   changes of enthalpy were higher for these ETs than for acyclic ETs (Fig. 6 in comparison to Fig. 7). The ETs

242   can be classified into two groups based on the strength of their interactions with gelatin. i) Seven ETs with a

243   glucopyranose core, *i.e.* tellimagrandin I, tellimagrandin II, agrimoniin, gemin A, sanguiin H-6, lambertianin

244   C, and roshenin C had stronger interactions with gelatin (Figs. 5 and 6) and the thermodynamic binding

245   parameters could be estimated for these interactions (Tables 4 and 5). ii) Four acyclic ETs, i.e. castalagin,

246   vescalagin, castavaloninic acid, and vescavaloninic acid, had no interaction or very weak interactions with

247   gelatin (Fig. 7) and no fits or thermodynamic binding parameters were obtained. For both BSA and gelatin,

248   geraniin is an exception to this rule, where it behaved more like the acyclic ETs and for gelatin no binding

249   parameters could be obtained.

250   In general, the interactions between gelatin and ETs are independent of ET concentration as

251   also seen to the interaction between ETs and BSA. However, there seems to be some exceptions, see for

252   example geraniin in Fig. 5C which shows the ITC data for the titration of geraniin into gelatin solutions of

253   varying concentrations (two replicates for 20 µM gelatin, 30 µM gelatin and 40 µM gelatin). The shapes of

254   the isotherms are completely different in comparison to those of other ETs with a glucopyranose core.

255   Typically the interaction between the protein and ET gets smaller with increasing injections (molar ratio) as

256   the saturation of the binding site on the protein occurs (Fig. 6 shows example of this usual behavior). However,

257   initially at low molar ratio the interaction between gelatin and geraniin gets stronger when more geraniin is

258   added (Fig. 5C) and the shape of the interaction changes with the increasing protein concentration. The

259   biphasic shape of the isotherms and dependence on protein concentration are similar to the ITC isotherms of

260   SDS titration into lysozyme.[43] There is a gradual increase in the binding enthalpy that reaches a plateau region,

261   with a maximum enthalpy change of the binding of approximately – 20 kJ/mol, followed by a decrease in the

262   measured enthalpy change as the protein binding sites become saturated. This would suggest that initial binding

263   is co-operative in that the presence of pre-bound ET initially promotes the exothermicity of the interaction.

264   Also, the binding is stronger in a higher protein concentration solution which could indicate oligomerization

265   of the protein and more complex protein-ET intermolecular structures. Others have also observed biphasic

266   binding isotherms in cases where the ligand induces oligomerization of the protein.[44] It is possible given the

267   structure of gelatin that geraniin is able to alter its secondary structure to promote oligomerization. Although

268   less pronounced, the acyclic ETs (Fig. 7), particularly castalagin, appear to exhibit a similar behavior.

269   For the interaction between the ETs with a glycopyranose core and gelatin, there is a clear link

270   between strength of binding and oligomerization of the ETs. Generally weaker interactions observed for the

271   monomers (K_{a1} range 0.8-1.8 × 10^5 M^{-1}) compared to the dimers (K_{a1} = 1.5-13 × 10^5 M^{-1}), and a strongest

272   interaction seen for the trimer ET (K_{a1} = 19 × 10^5 M^{-1}, Table 5).

273   In summary, we had a selection of purified ETs and were able to determine their interactions

274   with selected proteins, i.e. BSA and gelatin, in addition to the thermodynamic parameters related to this

275   interaction. Given the importance of this interaction in defining the biological activities of these molecules and

276   the current difficulties in studying such systems, the use of purified tannins provides structure-function

277   information that has previously been difficult to derive from less purified tannin fractions. BSA and gelatin

278   model different aspects of protein structure; BSA as a globular well-characterized protein and gelatin as a

279   proline-rich random coil structure that are also exhibited in salivary proline-rich proteins. Our data clearly

280   shows a difference in protein binding behavior of ETs with cyclic and acyclic glucose cores, showing very

281   weak binding to the acyclic structures where the ET tends to have a less open structure and relatively rigid

282   conformation. Our data show that ETs with a glucopyranose structure are able to bind more strongly to the

283   protein. These observations were observed for binding to both proteins. As expected, the binding to gelatin

284   was stronger than to BSA which indicates the importance of the more flexible protein structure on tannin

285   binding behavior. The binding was also dependent on the oligomerization of the ET, with the larger ET binding

286   more strongly. It is clear that the ETs bind to multiple sites on the surface of the protein and those able to wrap

287   around the protein structure and increase foot-holds to the protein surface are able to bind more strongly. For

288   the ET-gelatin binding, a two-site binding model better described the interaction of the dimeric ETs and we

289   observe for all ET-protein interactions relatively high values for n; this shows that the ET-protein interaction

290   is not dependent on a specific binding domain but is less selective with regions of higher and lower binding

291   affinity that may be related to hydrophobicity or surface charged regions rather than specific tertiary binding

292   regions. Thus, it may be easier to consider these interactions as a non-selective adsorption behavior particularly

293   when considering the second-binding site.

294   In most cases, a tannins biological activity, for example as an anthelmintic compound, may be

295   defined by how it interacts with proteins. Our data showed that acyclic ETs with NHTP groups had weaker

296   interaction with proteins than the ETs with a glucopyranose core. Similarly, the presence of NHTP groups was

297   shown to be an important factor in the anthelmintic effects of ETs, as detected by a decrease in the inhibition

298   activity of ETs against egg hatching of *H. contortus*.[8] It is thus probable that these types of ITC experiments

299   described in this paper are able to reveal the possible significant or non-significant role of tannins in many

300   aspects related to tannin-ruminant interactions.

301

302   ABBREVIATIONS USED

303   BSA, bovine serum albumin; DAD, diode array detection; ESI, electrospray ionization; ET, ellagitannin;

304   HHDP, hexahydroxydiphenoyl; HPLC, high-performance liquid chromatography; ITC, isothermal titration

305   calorimetry; MS, mass spectrometry; NHTP, nonahydroxytriphenoyl; UPLC-, ultra-performance liquid

306   chromatography

307

308   ACKNOWLEDGMENTS

312

313   ASSOCIATED CONTENT

314   ***S Supporting Information**

315   The Supporting Information is available free of charge on the ACS Publications website at DOI: x.

316   Figures S1-S6. Examples of thermograms for the interaction of ellagitannins with BSA and gelatin. The

317   thermograms include the raw heat data (µJ/s) from where the control experiment is not subtracted.

318   Table S1. Estimated entropies for the interaction of ellagitannins with BSA and gelatin fitted by two-site and

319   one-site binding models.

320   (PDF)

321

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463   **Notes**

464   The authors declare no competing financial interest.

FIGURE CAPTIONS

Figure 1. Individual ellagitannins studied for their interactions with BSA and gelatin. A refers to gallic acid, DHHDP to dehydrohexahydroxydiphenoyl group, G to galloyl group, GOD to linking between a galloyl and an HHDP group, GOG to linking between two galloyl groups, HHDP to hexahydroxydiphenoyl group and NHTP to nonahydroxytriphenoyl group.

Figure 2. Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of monomeric ellagitannins with a glucopyranose core: tellimagrandin I (A), tellimagrandin II (B), and the ITC binding isotherms for the interaction of monomeric ellagitannin geraniin (C) with 20 µM BSA in two replicate measurements (■ and □) and 30 µM BSA (Δ) and 40 µM BSA in two replicates (× and +).

Figure 3. Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of the ellagitannin dimers with a glucopyranose core: agrimoniin (A), gemin A (B), sanguiin H-6 (C), and trimer lambertianin C (D) with 30 µM BSA, and the ITC binding isotherms for the interaction of dimeric ellagitannin roshenin C (E) with 20 µM BSA (■) and 30 µM BSA in two replicate experiments (Δ and ×).

Figure 4. ITC binding isotherms for the interaction of acyclic ellagitannins castalagin (A) with 10 µM BSA (■), 20 µM BSA (Δ) and 30 µM BSA (×), vescalagin (B) with 20 µM BSA (■) and 30 µM BSA in two replicate experiments (Δ and ×), castavaloninic acid (C) with 20 µM BSA (■) and 30 µM BSA in two replicate experiments (Δ and ×) and vescavaloninic acid (D) with 20 µM BSA (■) and 30 µM BSA in two replicate experiments (Δ and ×).

Figure 5. Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of ellagitannins with a glucopyranose core: tellimagrandin (A) I with 10 µM gelatin and tellimagrandin II (B) with 20 µM gelatin. In addition, ITC binding isotherms for the

interaction of monomeric ellagitannin geraniin (C) with 6 µM gelatin in two replicate measurements (■ and □) and with 10 µM gelatin (Δ) and 20 µM gelatin (×).

Figure 6. Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of ellagitannins with a glucopyranose core: agrimoniin (A), gemin A (B), sanguiin H-6 (C), lambertianin C (D), and roshenin C (E) with 20 µM gelatin.

Figure 7. ITC binding isotherms for the interaction of acyclic ellagitannins castalagin (A), vescalagin (B), castavaloninic acid (C), and vescavaloninic acid (D) with 6 µM gelatin (■), 10 µM gelatin (Δ) and 20 µM gelatin (×).

Table 1. The Individual Ellagitannins Tested, Their Purities Obtained by UPLC at 280 nm and

Electrospray Ionization Mass Spectrometric (ESI-MS) Identification

No.	Ellagitannin	Purity (%)	ESI-MS identification (*m/z*)	Literature
1	Tellimagrandin I	97.5	785 [M–H]⁻	[27,31]
2	Tellimagrandin II	97.9	937 [M–H]⁻, 301 [ellagic acid–H]⁻	[27,29]
3	Geraniin	98.3	951 [M–H]⁻, 933 [M–H₂O–H]⁻,	[32]
4	Castalagin	99.6	933 [M–H]⁻, 466 [M–2H]²⁻, 301 [ellagic acid–H]–	[30,31,33]
5	Vescalagin	94.1	933 [M–H]⁻, 915 [M–H₂O–H]⁻, 466 [M–2H]²⁻, 457 [M–H₂O –2H]²⁻, 301 [ellagic acid–H]⁻	[30,31,33]
6	Castavaloninic acid	99.6	1101 [M–H]⁻, 528 [M–COOH–H]²⁻	[34,35]
7	Vescavaloninic acid	95.6	1101 [M–H]⁻, 1083 [M–H₂O–H]⁻, 528 [M–COOH–H]²⁻, 519 [[M–H₂O–COOH–H]²⁻, 301 [ellagic acid–H]⁻	[34,35]
8	Agrimoniin	97.7	934 [M–2H]²⁻, 301 [ellagic acid–H]⁻	[25,29,31]
9	Gemin A	98.2	935 [M–2H]²⁻, 301 [ellagic acid–H]⁻	[25,39]
10	Sanguiin H-6	97.6	934 [M–2H]²⁻, 301 [ellagic acid–H]⁻	[25,40]
11	Roshenin C	93.8	783 [M–2H]²⁻, 301 [ellagic acid–H]⁻	[38]
12	Lambertianin C	95.6	934 [M–3H]³⁻, 301 [ellagic acid–H]⁻	[25,40]

Table 2. Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Monomeric Ellagitannins with BSA Fitted by Two-Site and One-Site Binding Models. The Values for Tellimagrandin I Have Been Previously Published.[22] SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

Two-Site	Tellimagrandin I	Tellimagrandin II
K_{a1} (M^{-1})	22188 ± 6280	8308 ± 6248
ΔH_1 (kJ mol^{-1})	-20 ± 3	-33 ± 5
n_1	6 ± 2	29 ± 1
K_{a2} (M^{-1})	1828 ± 1815	36 ± 13
ΔH_2 (kJ mol^{-1})	-10 ± 9	-28 ± 6
n_2	4 ± 1	110 ± 27
SD	12 ± 2	28 ± 6

One-Site	Tellimagrandin I	Tellimagrandin II
K_{a1} (M^{-1})	18403 ± 5052	7284 ± 5408
ΔH_1 (kJ mol^{-1})	-24 ± 3	-37 ± 6
n_1	6 ± 1	30 ± 1
SD	13 ± 4	25 ± 6

Table 3. Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Dimeric and Trimeric Ellagitannins with BSA Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

Two-Site	Agrimoniin	Gemin A	Sanguiin H-6	Lambertianin C
K_{a1} (M^{-1})	35687 ± 20734	16413 ± 3912	35360 ± 8262	156900 ± 16108
ΔH_1 (kJ mol^{-1})	-18 ± 4	-37 ± 1	-18 ± 1	-25 ± 2
n_1	16 ± 1	14 ± 1	17 ± 1	11 ± 1
K_{a2} (M^{-1})	686 ± 245	784 ± 176	1134 ± 273	3117 ± 4760
ΔH_2 (kJ mol^{-1})	-8 ± 1	-8 ± 2	-6 ± 1	-3 ± 1
n_2	46 ± 1	30 ± 1	94 ± 12	37 ± 13
SD	19 ± 3	31 ± 3	20 ± 2	20 ± 8

One-Site	Agrimoniin	Gemin A	Sanguiin H-6	Lambertianin C
K_{a1} (M^{-1})	17140 ± 5892	11470 ± 1223	13337 ± 4242	107180 ± 37045
ΔH_1 (kJ mol^{-1})	-24 ± 4	-45 ± 4	-26 ± 2	-28 ± 1
n_1	17 ± 1	14 ± 1	19 ± 1	11 ± 1
SD	20 ± 4	29 ± 3	21 ± 3	26 ± 1

Table 4. Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Monomeric Ellagitannins with Gelatin Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

Two-Site	Tellimagrandin I	Tellimagrandin II
K_{a1} (M^{-1})	175600 ± 28614	84207 ± 16299
ΔH_1 (kJ mol^{-1})	-14 ± 3	-56 ± 1
n_1	52 ± 19	31 ± 1
K_{a2} (M^{-1})	88693 ± 53165	7092 ± 3005
ΔH_2 (kJ mol^{-1})	-11 ± 2	-3 ± 2
n_2	120 ± 55	59 ± 6
SD	15 ± 2	55 ± 12

One-Site	Tellimagrandin I	Tellimagrandin II
K_{a1} (M^{-1})	7592 ± 1492	76143 ± 5888
ΔH_1 (kJ mol^{-1})	-39 ± 9	-59 ± 2
n_1	67 ± 31	31 ± 1
SD	36 ± 2	48 ± 11

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Table 5. Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Dimeric and Trimeric Ellagitannins with Gelatin Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

Two-Site	Agrimoniin	Gemin A	Sanguiin H-6	Roshenin C	Lambertianin C
K_{a1} (M^{-1})	169667 ± 43966	1275667 ± 114001	220500 ± 18163	150600 ± 35982	1866000 ± 268836
ΔH_1 (kJ mol^{-1})	-65 ± 5	-64 ± 3	-63 ± 4	-30 ± 4	-91 ± 7
n_1	18 ± 2	15 ± 1	16 ± 1	24 ± 2	11 ± 1
K_{a2} (M^{-1})	11267 ± 6161	42425 ± 11770	42197 ± 8074	42063 ± 22578	250 ± 65
ΔH_2 (kJ mol^{-1})	-10 ± 1	-17 ± 1	-12 ± 1	-57 ± 8	-22 ± 10
n_2	52 ± 3	21 ± 2	38 ± 2	30 ± 2	15 ± 2
SD	78 ± 22	45 ± 18	66 ± 20	57 ± 13	55 ± 14

One-Site	Agrimoniin	Gemin A	Sanguiin H-6	Roshenin C	Lambertianin C
K_{a1} (M^{-1})	74353 ± 10169	417533 ± 104665	72583 ± 14192	17007 ± 5878	1534000 ± 129526
ΔH_1 (kJ mol^{-1})	-76 ± 5	-81 ± 2	-78 ± 3	-57 ± 8	-93 ± 5
n_1	20 ± 2	16 ± 1	17 ± 1	30 ± 3	11 ± 1
SD	117 ± 20	88 ± 9	123 ± 12	96 ± 16	60 ± 7

Fig. 1.

HHDP group

$^4C_1$ glucose

Tellimagrandin I R^1 = OH, R^2 = R^3 = G
Tellimagrandin II R^1 = β-OG, R^2 = R^3 = G

DHHDP group

$^1C_4$ glucose

Geraniin R^1 = G, R^2 ~ R^3 = (R)-HHDP

Sanguiin H-6 R^1 ~ R^2 = (S)-HHDP
Roshenin C R^1 = R^2 = H

Acyclic glucose

NHTP group

Castalagin R^1 = R^3 = H, R^2 = OH
Vescalagin R^1 = OH, R^2 = R^3 = H
Vescavaloninic acid R^1 = OH, R^2 = H, R^3 = A
Castavaloninic acid R^1 = H, R^2 = OH, R^3 = A

m-GOG

Agrimoniin α, R^1 ~ R^2 = (S)-HHDP
Gemin A β, R^1 = R^2 = G
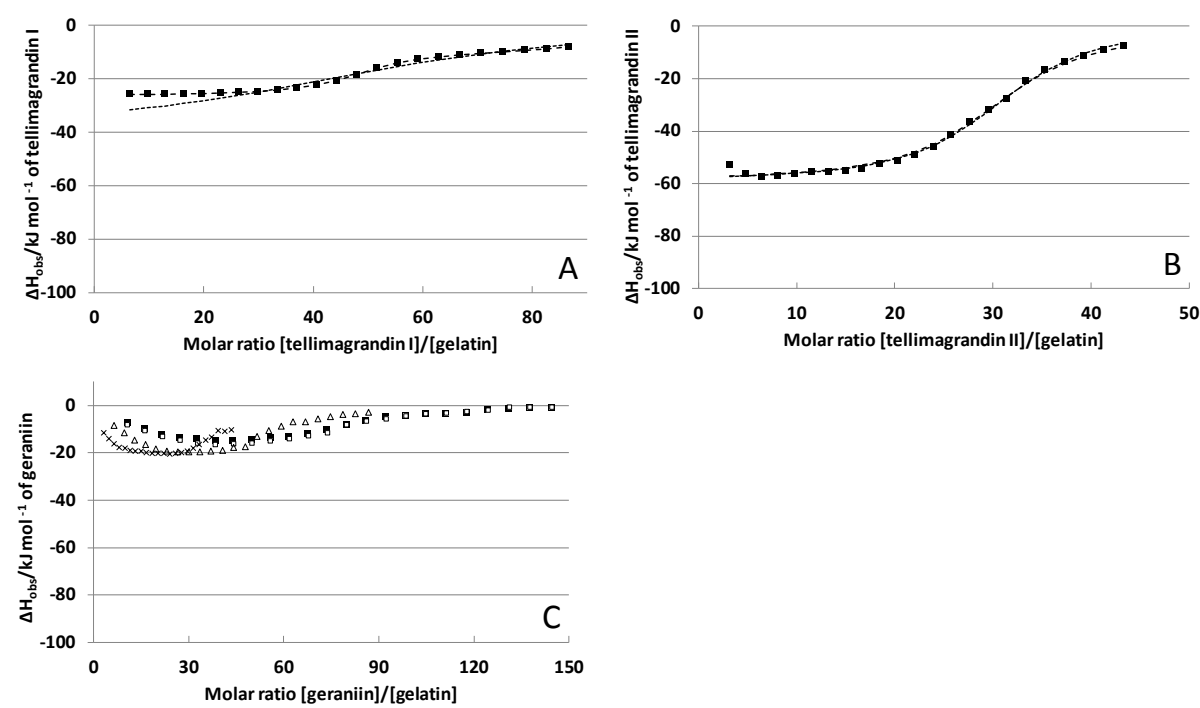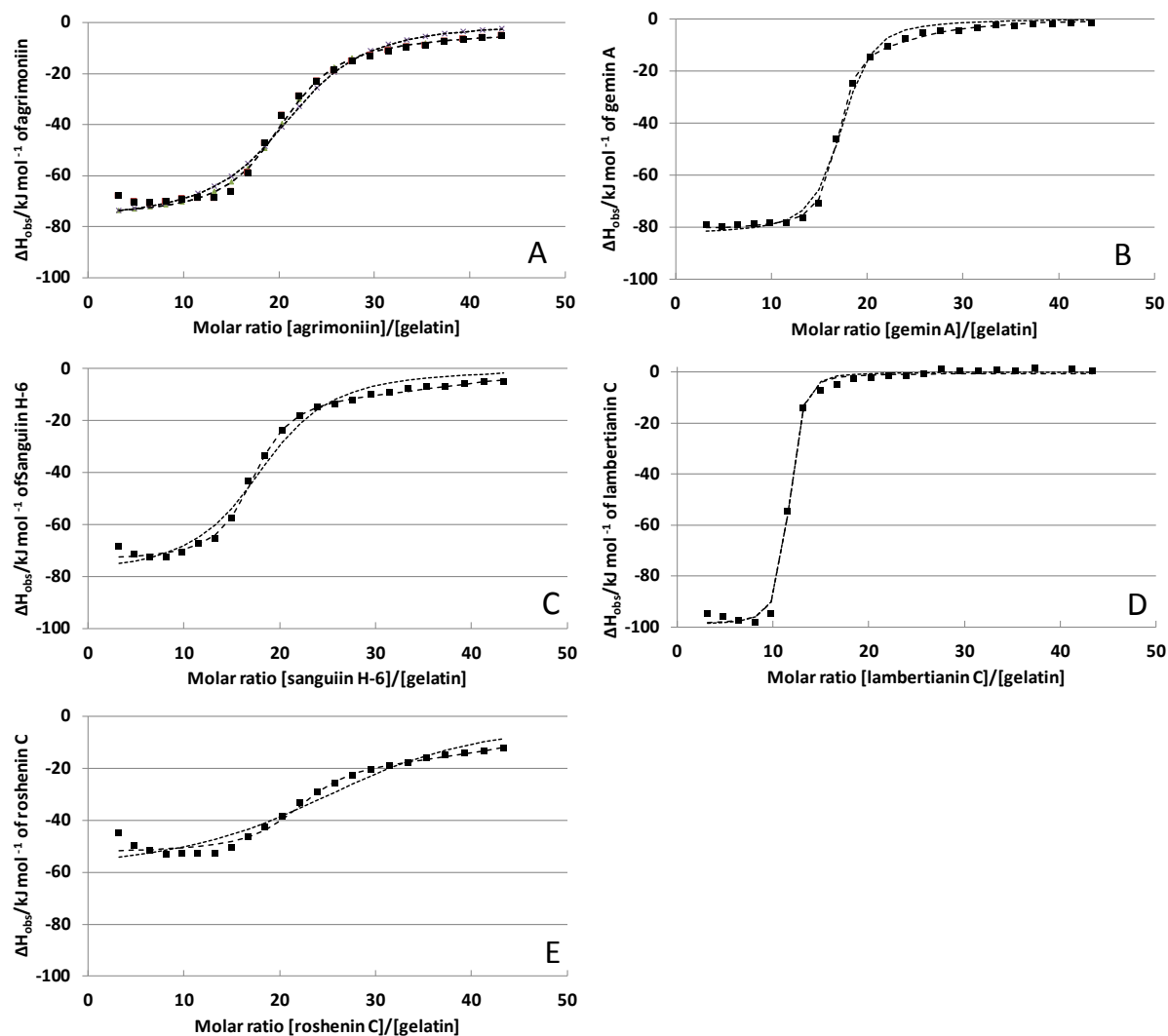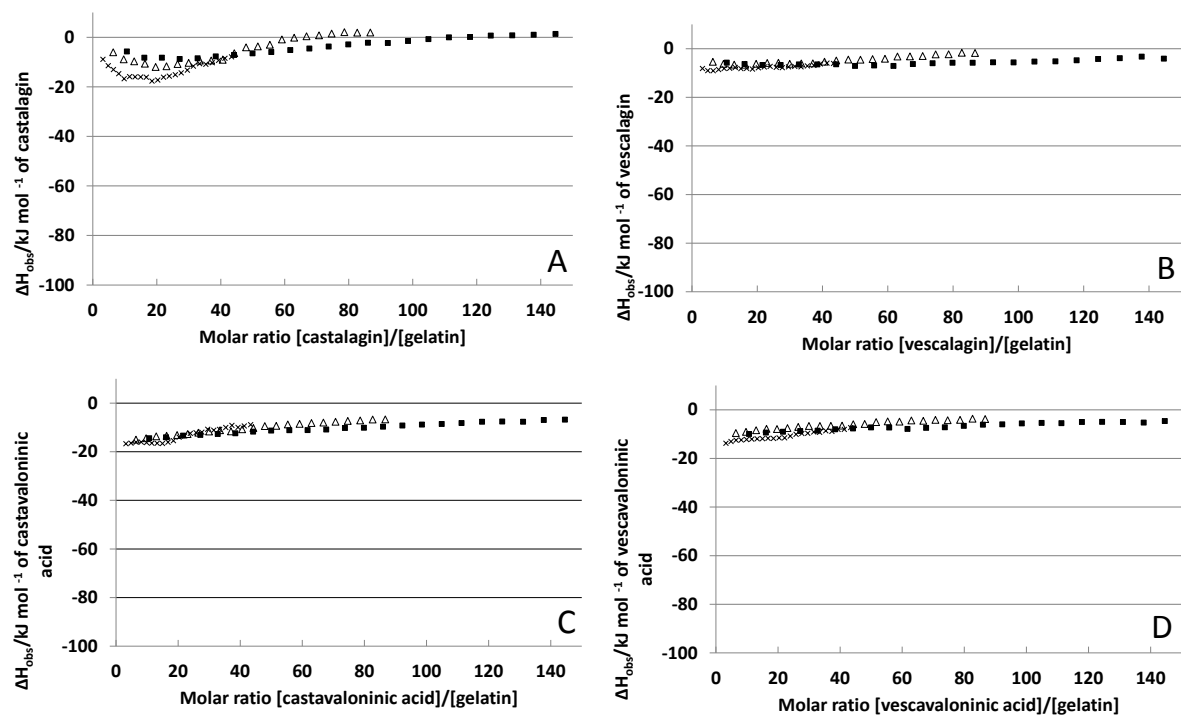
m-GOD

Lambertianin C R^1 ~ R^2 = (S)-HHDP

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Graphic for table of contents