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Synthesis and Biological Activity of Polygalloyl-Dendrimers as Stable Tannic Acid Mimics

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Abstract—Inspired by the structure of tannic acid, first- to third-generation dendrimers containing two, four, and eight galloyl moieties were synthesized. Stability, antioxidant activity and collagen cross-linking activity of the natural product and its dendrimer analogues were compared. The experimental results indicate that polygalloyl dendrimers might be used as new lead compounds to improve the long-term healing characteristics of burn wounds.

Advances in surgical techniques and the general care of burn patients over the past century have resulted in a strong decrease in the mortality due to thermal injury. Attention now has to be focused on the development of therapeutic regimens that can improve the cosmetic outcome of the treatment of extensively burned patients. One of the most promising candidates in this respect is tannic acid (Fig. 1), a mixture of polygalloylglucose esters or gallotannins, which is found in high concentrations in the galls of *Rhus* and *Quercus* species.[1] It has been topically applied with success in the past, in particular in the period between 1920 and 1940 just before the introduction of penicillin.[2] In the early 1990s, the beneficial effects on wound healing were further substantiated as it was shown that tannic acid positively influenced inflammation and processes involved in granulation tissue formation, re-epithelialization, and scar-tissue formation.[3,4]

However, a major shortcoming of tannic acid is its relative instability in pharmaceutical formulations. Facile hydrolysis of its meta-depsidic galloyl ester bonds results in the release of gallic acid, besides other low molecular weight constituents.[5] Since elevated gallic acid levels have recently been related to the hepatotoxic effects ascribed to the tannic acid treatment of burns in the past,[2,6] we considered it worthwhile to synthesize structurally related analogues that combine a higher stability with a similar biological activity.

To achieve this objective we have chosen to synthesize first-, second-, and third-generation dendrimers containing two, four, and eight galloyl moieties, respectively. Dendrimers were used with a 3,5-di(2-aminoethoxy)benzoic acid repeating unit.[7] They were previously used as multivalent scaffolds[8] for carbohydrate ligands which resulted in enhanced binding to cholera toxin and galectins.[9] A convergent synthesis was used that started with the benzyl protection of the three hydroxyl groups of the starting material methyl gallate **1** (Scheme 1). After saponification of the methyl ester

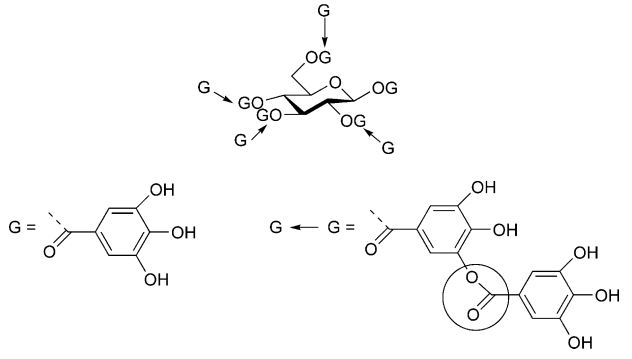

Figure 1. General structural representation of tannic acid. On average, five to eight gallic acid residues are attached to the glucose-nucleus at the positions shown, depending on the plant source. The larger structures contain the encircled labile meta-depsidic galloyl ester bond.

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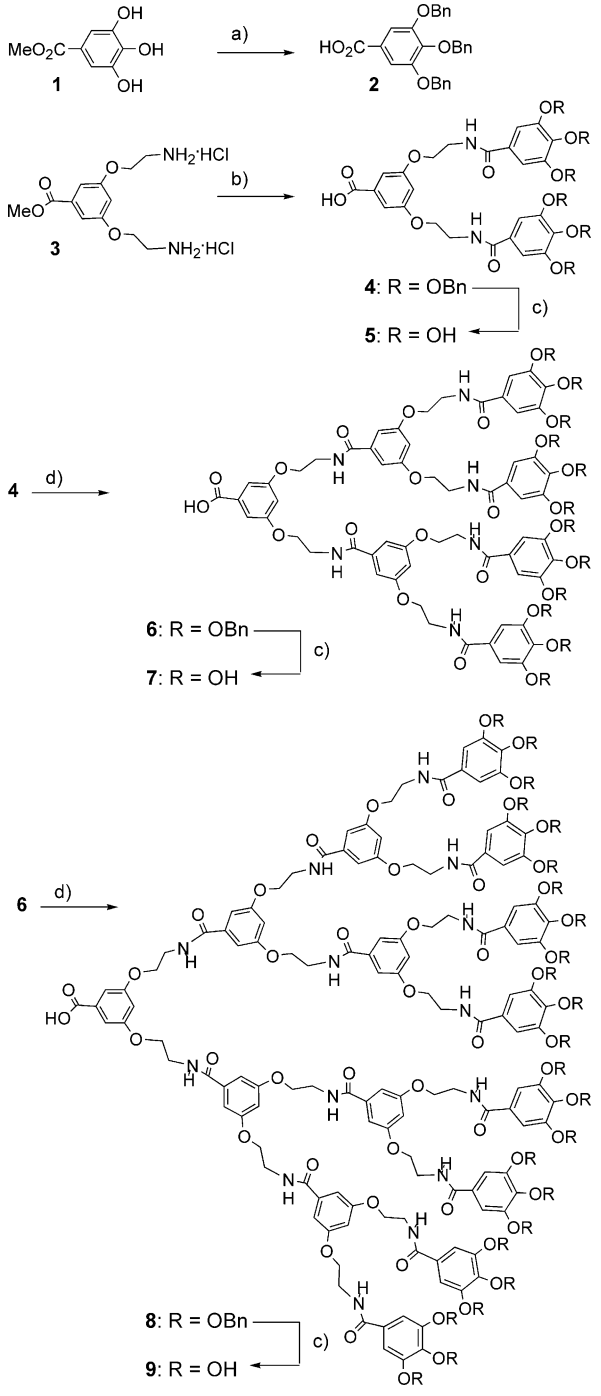
the resulting carboxylic acid **2** was coupled to the first generation dendrimer, diamine **3**,[7] using the peptide coupling reagent BOP[10] in the presence of base in dichloromethane. The coupling product was subjected to basic conditions to hydrolyze the methyl ester to produce compound **4**. Removal of the benzyl groups by hydrogenation yielded the di-galloyl compound **5**. In order to create the larger structures, **4** was coupled to dendrimer monomer **3**, which after removal of methyl ester functionality and benzyl groups gave the tetra-galloyl dendrimer **7**. Repeating the sequence from the benzyl protected **6** resulted ultimately in octa-galloyl dendrimer **9** and completed the series.[11]



Scheme 1. Synthesis of the galloyl dendrimers. Reaction conditions: (a) (i) BnCl, K_2CO_3, DMF, 100 °C, 14 h, 81%; (ii) NaOH, H_2O/MeOH/dioxane, 95%; (b) (i) **2**, BOP, *i*Pr_2NEt, CH_2Cl_2, 82%; (ii) NaOH, H_2O/MeOH/dioxane 92%; (c) H_2, Pd/C, DMF, quant for **5**, 51% for **7**, 91% for **9**; (d) (i) **3**, BOP; *i*Pr_2NEt, CH_3CN/DMF; (ii) NaOH, H_2O/MeOH/dioxane, 78% for **6**, 36% for **8**.

The higher molecular weight polygalloylglucose esters in tannic acid contain a pentagalloylglucose nucleus to which additional galloyl groups are attached by depsidic linkages.[12] Due to the lability of these linkages, tannic acid in solution is easily degraded. Stability-testing was performed with ethanolic solutions of a highly purified tannic acid product (Brewtan™, Omnichem B.V., Wetteren, Belgium; 3.6 mM) and **9** (0.6 mM) which were kept for 2 and 4 weeks. Degradation was monitored using a modified version of the HPLC-system described by Beasley et al.[13] In agreement with literature data,[5] degradation of tannic acid was characterized by the appearance in the HPLC-chromatogram of peaks corresponding with mono-, di-, and trigallic acid derivatives (data not shown). In comparison, the stability of the synthetic analogue **9** was found to be far superior. Even after 4 weeks in solution, only the single peak of **9** was detectable. No additional peaks of degradation products were present (data not shown).

Two biological assays were used in this study. One assay determines antioxidant activity and another provides information about the collagen cross-linking abilities. The assays were selected because they can provide important information with which it seems possible to predict the in vivo efficacy of new drugs for the treatment of burns. Thus, the tannic acid product which improved wound healing in an animal-experimental as well as clinical setting,[3,4] is also found to be highly active in these assays.

The antioxidant activity of Brewtan, gallic acid (Carl Roth GmbH, Karlsruhe, Germany), and the synthetic analogues **5**, **7**, and **9**, was determined as the decolorization of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma Chemical Co., St. Louis, MO, USA).[14,15] Antioxidant activity varied considerably among the compounds tested (Table 1). Tannic acid was most active in this respect. The antioxidant activity of **9** was slightly but significantly lower and further declined

Table 1. Antioxidant and collagen cross-linking activity of tannic acid, gallic acid, and the synthetic analogues **5**, **7**, and **9**

Compd	Antioxidant activity ED_{50} (μM)[a,b]	Collagen cross-linking T_s (°C)[a,c]
Tannic acid	2.9 (±0.2)	59.5 (±0.3)[e]
Gallic acid	14.3 (±3.6)[d]	57.5 (±0.2)[d,e]
5	13.7 (±1.0)[d]	58.4 (±0.3)[e]
7	7.4 (±0.6)[d]	57.9 (±0.1)[d,e]
9	4.7 (±0.4)[d]	57.0 (±0.1)[d]

[a]Values are means of five experiments, standard error of the mean is given in parentheses.
[b]Effective dose giving 50% decolorization of DPPH.
[c]Temperature at which collagen shrinkage is initiated. Values depicted were obtained with sample concentrations of 4 mM in 85% glycerol. T_s for untreated collagen matrix is 56.4 (±0.2)°C.
[d]Significantly different from tannic acid, $p < 0.05$.
[e]Significantly different from control, $p < 0.05$.

in the series of **7**, **5**, and gallic acid. However, these differences can be totally accounted for by the number of galloyl moieties per molecule and the relative size of the backbone to which these galloyls are attached.[16]

Effects on cross-linking of collagen were assessed according to the method of Heijmen et al.[17] (Table 1). Compounds were dissolved in 85% glycerol and incubated for 48 h at room temperature with purified skin collagen matrices. The degree of collagen cross-linking was determined by the hydrothermal shrinkage temperature (T_s). The T_s denotes the change in molecular conformation of collagen from triple helix to random coil and will be increased when cross-linking has taken place.[18] The strongest increase in T_s was observed for the collagen matrices pretreated with tannic acid. For **5**, elevation of the T_s was less pronounced but not significantly different from tannic acid. In comparison, the cross-linking capacity of **7** and **9** lagged behind, the T_s of collagen matrices pretreated with **9** not even being distinct from the controls without tannins/dendrimers. These results fit within the concept for tannic acid–collagen interactions as proposed by Haslam.[19] In this model, pentagalloylglucose as the prototype molecule representative for tannic acid, has in its most favored conformation a disc-like shape with a thickness of approximately 0.7 nm and a diameter of approximately 2.1 nm. These dimensions are ideally suited to infiltrate in the gaps between the tropocollagen molecules that compose the collagen fibrils. Hydrophobic effects are thought to initiate the association with hydrogen bonding acting to reinforce the structure. The decline in collagen cross-linking capacity going from the first- to third-generation dendrimers is consistent with the disc model. Bis-galloyl compound **5** is more likely to adopt a flat conformation than the more three-dimensional **7** and **9**. Alternatively, spatial contraints may simply not allow the accommodation of the significantly larger structures **7** and **9**, as compared to **5** or tannic acid. Furthermore the difference in hydrophobicity between the galloyl dendrimers cannot be ruled out as a determining factor either considering the importance of hydrophobicity in the initiation of the association.

As shown in this paper, the synthetic analogues **5**, **7**, and **9**, retain a satisfactory, albeit lower, biological activity in comparison to tannic acid, but have the advantage of a much enhanced stability. Therefore, polygalloyl-dendrimers may serve as potential leads for the development of new topical drugs to be used in burn wound treatment. Considering the importance of both the number of galloyl moieties and possibly molecular size for the biological activity, it might finally be of interest to search for other synthetic analogues of tannic acid in which the backbone is reduced in dimension but still can accomodate a maximal load of gallic acid residues.

Acknowledgements

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11. ^{13}C NMR data, see ref 9a for numbering of the dendrimer backbone, Gal = galloyl moiety. **5**: ^{13}C NMR (CD_3OD, 75.4 MHz) δ 171.7, 170.8 ($C{=}O^{I,\ II}$), 161.1 ($C^{3/5}$), 146.6 (Gal-$C^{3/5}$), 138.1, 136.8 (Gal-C^4, C^1), 126.0 (Gal-C^1), 109.2 (C^1), 107.9 (Gal-$C^{2/6}$), 106.3 (C^4), 67.7 (CH_2^a), 40.5 (CH_2^b); ESI-MS: $m/z = 545.1$ $[M + H]^+$ (100%), 567.0 $[M + Na]^+$; **7**: ^{13}C NMR (CD_3OD, 75.4 MHz) δ 170.8, 170.2 ($C{=}O^{I,\ II,\ III}$), 161.3, 161.1 ($C^{3/5}$, $C^{3'/5'}$), 146.7 (Gal-$C^{3/5}$), 138.1, 137.5 (Gal-C^4, C^1, $C^{1'}$), 126.0 (Gal-C^1), 109.4, 107.2, 106.1, 105.7 ($C^{2/6}$, $C^{2'/6'}$, C^4, $C^{4'}$), 107.9 (Gal-$C^{2/6}$), 67.8 ($CH_2^{a'}$), 67.5 (CH_2^a), 40.8 (CH_2^b), 40.5 ($CH_2^{b'}$); MALDI-TOF-MS: $m/z = 1293$ $[M + H]^+$ (100%); **9**: ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 167.0, 166.2 ($C{=}O^{I,\ II,\ III,\ IV}$), 159.8 ($C^{3''/5''}$), 159.7 ($C^{3'/5'}$), 159.4 ($C^{3/5}$), 145.8 ($C^{3/5}$), 136.7, 136.6, 136.5 (Gal-C^4, C^1, $C^{1'}$, $C^{1''}$), 124.8 (Gal-C^1), 107.8, 106.2, 104.4, 104.1 ($C^{2/6}$, $C^{2'/6'}$, $C^{2''/6''}$, C^4, $C^{4'}$, $C^{4''}$), 107.1 (Gal-$C^{2/6}$), 66.6 ($CH_2^{a''}$), 66.5 ($CH_2^{a'}$), 66.3 (CH_2^a), ($CH_2^{b,b',b''}$ signals obscured by DMSO-d_6 signals).
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CH_3COOH and mobile phase B: $EtOH/C_4H_8O$ (3:1) with 0.5% CH_3COOH, gradient: 0–60 min: isocratic 27% B, 60–70 min: 27→100% B, 70–75 min: isocratic 100% B, 75–80 min: 100→27% B, 80–90 min: isocratic 27% B, total flow: 1 mL/min. Injection volume: 10l. Detection: 254 and 280 nm.

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15. Test compounds were serially diluted (0.1–180 μM) in flat-bottom microtiter plates and incubated with a 250 μM DPPH solution in 75% ethanol for 15 min at room temperature. Subsequently, absorbance was read at 550 nm using an automatic ELISA-reader (SLT Labinstruments, Salzburg, Austria). ED_{50} values were calculated by linear regression analysis of the absorbance curves obtained for each compound.

16. Since the galloyl groups present in tannic acid and **5**, **7**, and **9**, are considered to be the parts in these molecules to accept the free radical from DPPH, it is possible to predict antioxidant activities on basis of the experimental ED_{50}-value of gallic acid using the formula: $ED_{50}(x) = ED_{50}(\text{gallic acid})/n(x)*R_{MW}(x)$, in which $n(x)$ is the total number of galloyl groups in compound x and $R_{MW}(x)$ the ratio between the molecular weight of all galloyl groups present in compound x and the total molecular weight of this compound. The latter ratio is introduced in the formula to correct for the differences in weight of the backbone structure to which the galloyl groups are attached. This scaffold is significantly larger in the dendrimeric analogues yielding R_{MW}-values of 0.88, 0.56, 0.47, and 0.44 for tannic acid and **5**, **7**, and **9**, respectively. ED_{50}-values calculated according to the above formula are 2.0 (±0.5), 12.7 (±3.2), 7.5 (±1.9), and 4.1 (±1.0) μM for tannic acid and **5**, **7**, and **9**, respectively. These values do not differ significantly from the experimental data.

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