Synthesis, Antibacterial Activity and Cytotoxicity of Novel Janus Peptide Dendrimers

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Abstract: In an attempt to find new antibacterial agents, a series of well-defined Janus peptide dendrimers, which feature multiple anionic groups and amphiphilic structure, were synthesized and characterized in detail. The antibacterial activities of all the synthesized dendrimers were tested and screened by using the two-fold serial dilution method. Several compounds showed activity against *E. coli*, *S. aureus*, methicillin-resistant *S. aureus*, and *E. faecalis*. Further cytotoxicity assays showed that the antibacterial dendrimers were nontoxic against HEK293.

Key words: Janus dendrimer, peptide dendrimer, amphiphilic, antibiotics, antibacterial activity

Dendrimers are artificial macromolecules that have monodisperse and highly branched structures with well-defined three-dimensional architecture. With the development of macromolecules, dendrimers have been widely applied in medical and biomedical fields such as drug delivery[1] and gene delivery.[2] Moreover, some dendrimers have inherent pharmacological activities such as antibacterial, antiviral, and antitumor activities.[3] Because of the dramatic structural differences between dendrimers and traditional small-molecule drugs, dendrimers provide new and distinct molecules that might be used to address problems of drug resistance, especially in the antibacterial drug field.

Currently, a number of dendrimer classes (e.g., glycol-dendrimers,[4] *m*-terphenyl surfaced dendrimers[5]) have been reported as antibacterial agents. However, research on antibacterial dendrimers has mainly focused on cationic dendrimers, such as PAMAM dendrimers and their PEGylated derivates,[6] quaternary ammonium functionalized PPI dendrimers,[7] amine and ammonium terminated carbosilane dendrimers,[8] and peptide dendrimers that are rich in Arg and/or Lys sequences.[9] The cationic dendrimers show antibacterial activity because they are able to adhere to and damage the anionic bacterial membrane so as to cause bacterial lysis.[7a] Meanwhile, the use of cationic dendrimers in biological systems is constrained because of the inherent toxicity, which is also attributed to the interaction of the surface cationic charge of the dendrimers with negatively charged biological membranes in vivo.[10]

Janus dendrimers,[11] which contain two different functionalized segments on opposite sides, have been widely studied for their self-assembly properties,[12] thermal behavior,[13] and their application to drug delivery.[14] Recently, Grinstaff et al. reported a series of anionic Janus dendrimers that are composed of myristic acid and multivalent anions, and several of these dendrimers exhibited antibacterial activity with minimal eukaryotic cell cytotoxicity.[15] Clearly different from cationic antibacterial dendrimers, these anionic dendrimers exhibited striking selectivity in their cytotoxicity toward a prokaryotic bacterium compared to a eukaryotic human cell. Although the specific mechanism of the antibacterial activity of these anionic dendrimers is still unknown, it is generally considered to involve imitating detergent activity.[3d] Accordingly, studies with this kind of anionic dendrimer expanded the understanding of dendrimers as antibacterial agents. Subsequently, Tulu et al. also reported anionic dendrimers that displayed antibacterial activity against both Gram-positive and Gram-negative bacteria.[16]

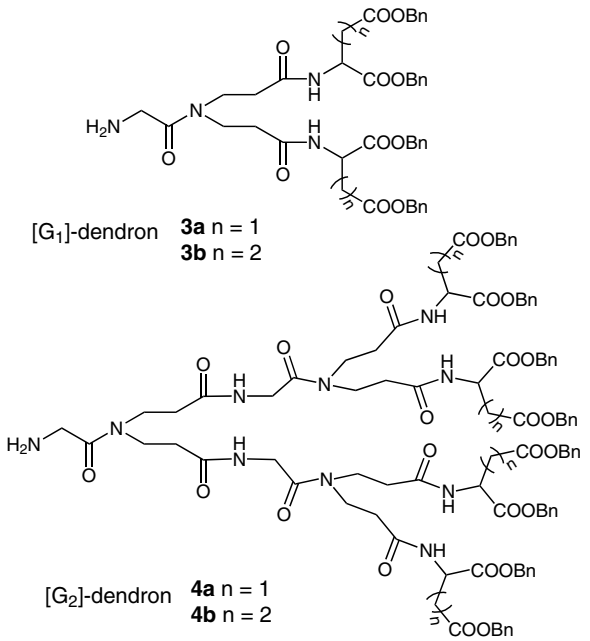

Figure 1 [G_1] and [G_2] Dendritic Asp/Glu[17]

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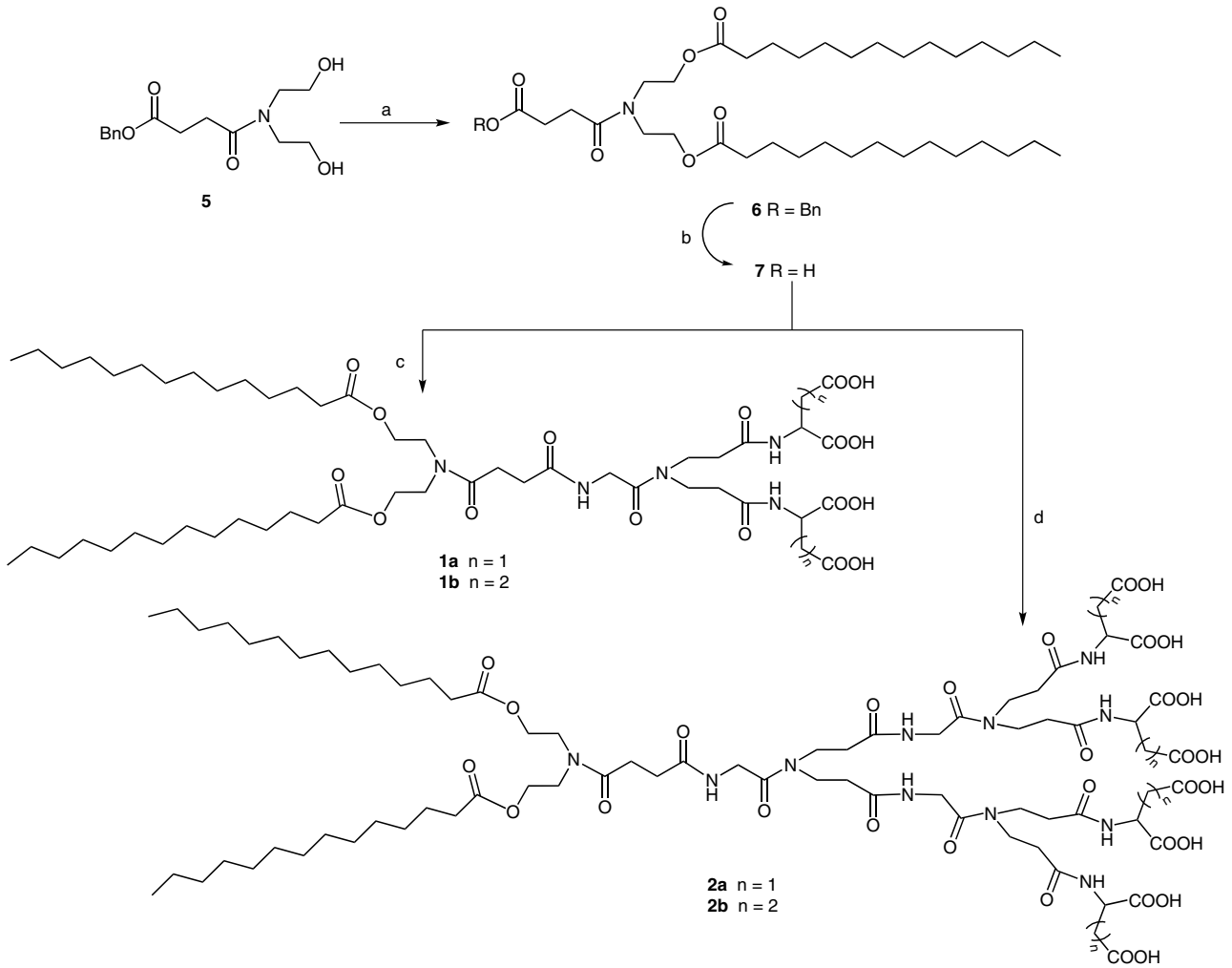
In our previous work, dendritic L-aspartic acid (L-Asp) and L-glutamic acid (L-Glu) were prepared as drug delivery systems (Figure 1).[17] We considered that the application of these peptide dendrons could be expanded to construct anionic dendrimers as antibacterial agents because (i) L-Asp and L-Glu are promising surface blocks to supply multivalent anions; (ii) peptide dendrimers based on natural amino acids are generally biocompatible and immunocompatible.[18] In this study, we synthesized a series of Janus peptide dendrimers as Grinstaff type anionic dendrimers. The amphiphilic Janus dendrimers contained myristic acid as the lipophilic end, and negatively charged native amino acids as the hydrophilic end. The antibacterial activity and cytotoxicity of the new dendrimers were evaluated through two-fold serial dilution method and MTT assays, respectively.

First, the two types of the functional dendrons were prepared by a convergent approach. The [G_1] and [G_2] dendritic Asp/Glu were synthesized according to our previous report.[17] The [G_1]-dendritic myristic acid **6** was prepared by utilizing EDCI and DMAP as the coupling reagents between core **5** and myristic acid. Subsequently, activation of the focal point was achieved by removal of the benzyl group from **6** by catalytic hydrogenolysis, giving compound **7** (Scheme 1)

The synthetic procedure used to access the target dendrimers involved two steps: assembly of the two dendrons together, and removal of the protecting groups (Scheme 1). Generally, the coupling of two different dendrons by connecting their cores is challenging because of the steric hindrance at dendron focal points, which may render the coupling inefficient and then lead to low yields. In order to assemble the two dendrons effectively, several coupling reagents, such as EDCI/1-hydroxy benzotriazole (HOBt), *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/DIPEA and isobutyl chloroformate (IBCF)/NMM were tried; it was found that use of the IBCF/NMM system provided the best yields. Finally, after removal of the protected groups (benzyl) by catalytic hydrogenolysis, the target molecules **1a**/**b** and **2a**/**b** were obtained. Quantitative coupling was proven by ^{1}H NMR spectroscopic analysis, which revealed reso-



Scheme 1 Preparation of the Janus Dendrimer. *Reagents and conditions*: (a) myristic acid, EDCI, DMAP, CH_2Cl_2, r.t., 24 h, 82%; (b) H_2, Pd/C (10 wt%), CH_2Cl_2–MeOH, r.t., 8 h, 99%; (c) i. **3a**/**3b**, IBCF, NMM, THF, –15 °C to r.t., 24 h; ii. H_2, Pd/C (10 wt%), MeOH, r.t., 24 h (**1a**: 75%; **1b**: 72% for two steps); (d) i. **4a**/**4b**, IBCF, NMM, THF, –15 °C to r.t., 24 h; ii. H_2, Pd/C (10 wt%), MeOH, r.t., 24 h (**2a**: 64%; **2b**:61% for two steps).

nance signals that could be assigned to the two opposite sides. For Asp-grafted dendrimers **1a** and **2a**, the structures were confirmed by the appearance of peaks at δ = 4.50 ppm (Asp-α-C*H*) and the peaks at δ = 0.85, 1.23, and 1.49 ppm (signals of myristic acid). For Glu-grafted dendrimers **1b** and **2b**, the peaks of Glu-α-CH were overlapped with those of the dendritic skeleton protons; in this case the peaks at δ = 1.75 ppm (Glu-β-CH_2) could be used to compare with the peaks of myristic acid, which confirmed the perfect joining. Furthermore, the formation of well-defined dendrons and dendrimers was further verified by ESI MS analysis, and elemental analysis was also in good agreement with those of the target structures.[19]

The antibacterial activity of Janus peptide dendrimers against strains of four pathogenic bacteria, including two standard strains (*E. coli* and *S. aureus*) and two clinical strains (methicillin-resistant *S. aureus* MRSA and *E. faecalis*), were determined by applying the two-fold serial dilution method.[20] The minimum inhibitory concentration (MIC) values are presented in Table 1. Dendrimers **1a** and **1b** were inactive even when the concentration was increased to the highest level (0.256 mM), while **2a** and **2b** showed moderate activity against most of the tested pathogens. We supposed that the dramatically different antibacterial activities between **1a**/**b** and **2a**/**b** were caused by the difference of amphipathicity. For various known antibacterial agents, either macromolecule (dendrimer and polymer) or small molecular compounds, suitable amphipathicity was important for the antibacterial activity because the amphiphilic structure acted through perturbation and disruption of the prokaryotic membrane.[21] For the dendrimers studied in this paper, while the lipophilic dendrons are the same, **2a**/**b** (with [G_2]-Asp/Glu dendron) possessed a higher number and more condensed hydrophilic groups than **1a**/**b** (with [G_1] -Asp/Glu dendron). Presumably, the hydrophilic/lipophilic ratio of **2a** and **2b** provided appropriate amphipathicity for the antibacterial activity. Moreover, **2a** was the most active compound; revealing an MIC value of 0.016 mM against MRSA compared with 0.128 mM for Cefotaxime and more than 0.256 mM for Ampicillin.

Cytotoxicity is one of the most important factors to be considered in selecting dendrimers for biomedical applications. The toxicity of **2a** and **2b** against HEK293 cells was evaluated by MTT assays[20] and the results are shown in Figure 2. The assays demonstrated that neither **2a** nor **2b** was significantly toxic against HEK293 cells at concentrations up to 1 mM. The synthesized anionic dendrimers based on natural amino acids were nontoxic, as expected.

In summary, we synthesized a series of Janus peptide dendrimers that showed amphipathicity due to the multivalent anions and alkyl chains. The antibacterial activity of these dendrimers against strains of four pathogenic bacteria was tested in vitro. It was found that **2a** and **2b** were moderately active against the majority of the tested pathogens. Especially, **2a** revealed an MIC value of 0.016 mM against MRSA. Moreover, cell viability studies showed that neither **2a** nor **2b** exhibited significant cytotoxicity against HEK293 cells at concentrations up to 1 mM. The information presented here may be used to expand the understanding of dendrimers as antibacterial agents and for the design of new compounds with improved antibacterial potential.

Table 1 Antibacterial Activity of Janus Dendrimers

Compounds	MIC (mM)			
	E. coli[a]	*S. aureus*[b]	MRSA[c]	*E. faecalis*[d]
1a	>0.256	>0.256	>0.256	>0.256
1b	>0.256	>0.256	>0.256	>0.256
2a	0.064	0.064	0.016	0.128
2b	0.032	0.128	0.128	0.256
Cefotaxime	0.001	0.002	0.128	0.002
Ampicillin	0.001	0.001	>0.256	0.001

[a] *Escherichia coli*ATCC25922.
[b] *Staphyloccocus aureus* ATCC29213.
[c] Methicillin-resistant *Staphylococcus aureus*. Clinical isolated strain from West China Hospital, Chengdu, P. R. of China.
[d] *Enterococcus faecalis*. Clinical isolated strain from West China Hospital, Chengdu, P. R. of China.

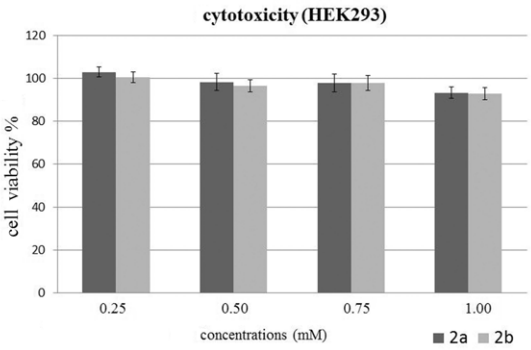


Figure 2 Cytotoxicity of dendrimers against HEK293 cells over a 24 hour incubation period determined by MTT assay. Cell viability was expressed as a percentage of the control cell culture. Values are represented as mean ± SD (n = 3).

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett. Included are detailed synthetic procedures and characterization data, as well as methods for antibacterial and toxicity experiments.

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(19) **Synthesis of Janus Dendrimers; Typical Procedure for 2a**: Compound **7** (313 mg, 0.5 mmol) dissolved in anhyd THF (25 mL) was cooled to –15 °C, and NMM (10 mmol) and IBCF (10 mmol) were added. After stirring for 10 min, **4a** (900 mg, 0.5 mmol) dissolved in anhyd THF (10 mL) was added dropwise. The reaction mixture was vigorously stirred at r.t. for 24 h, then the solution was concentrated under vacuum, and the residue was taken up in EtOAc (30 mL) and washed with 1 M HCl (10 mL), 1 M $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried over anhyd Na_2SO_4. After concentration, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to obtain a white waxy solid. Pd/C (10%, 100 mg) was added to a solution of the obtained white waxy solid in MeOH–CH_2Cl_2 (30 mL, 3:1 v/v). The reaction mixture was stirred under a hydrogen atmosphere for 24 h, filtered through a membrane filter, and concentrated under reduced pressure to afford **2a** (539 mg, 64%) as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.83–0.87 (t, J = 6.4 Hz, 6 H, 2 × CH_3), 1.23 (br s, 40 H, myristic acid 20 × CH_2), 1.48–1.49 (m, 4 H, myristic acid-β-CH_2 × 2), 2.24–2.35 (m, 8 H, myristic acid-α-CH_2 × 2 + Asp-β-CH_2/2 × 4), 2.38–2.59 (m, 16 H, succinic acid CH_2 × 2 + NCH_2CH_2CONH × 6), 2.63–2.70 (m, 4 H, Asp-β-CH_2/2 × 4), 3.41–3.60 (m, 16 H, NCH_2CH_2O × 3 + NCH_2CH_2CONH × 3), 3.99 (br s, 6 H, Gly-CH_2 × 3), 4.05–4.18 (m, 4 H, NCH_2CH_2O × 2), 4.48–4.54 (m, 4 H, Asp-α-CH × 4), 7.89 (br s, 1 H, CONH), 7.96 (br s, 1 H, CONH), 8.10 (br s, 1 H, CONH), 8.25 (br s, 1 H, CONH), 8.27 (br s, 1 H, CONH), 8.39 (br s, 1 H, CONH), 8.41 (br s, 1 H, CONH), 12.64 (br s, 8 H, Asp-COOH × 8). MS (ESI): m/z $[M + Na + H]^+$ calcd for $C_{76}H_{123}N_{11}O_{31}$: 1685.84; found: 1709.5. Anal. Calcd for $C_{76}H_{123}N_{11}O_{31}$: C, 54.11; H, 7.35; N, 9.13; O, 29.40. Found: C, 54.03; H, 7.26; N, 9.01; O, 29.31.
Detailed characterization data of **1a**, **1b**, and **2b** are provided in the Supporting Information (see S1 for details).

(20) Methods for antibacterial and toxicity experiments are given in the Supporting Information (see S2 for details).

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