Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2785–2788

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A DIRECT METHOD FOR THE FORMATION OF PEPTIDE AND CARBOHYDRATE DENDRIMERS

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Received 14 June 1999; accepted 19 August 1999

Abstract: Two new methods for the modification of PAMAM dendrimers have been developed which allow the covergent synthesis of either peptide or carbohydrate-bearing dendrimer molecules. Both methods involve condensation between hydroxylamino nucleophiles and appropriate carbonyl-bearing reaction partners.

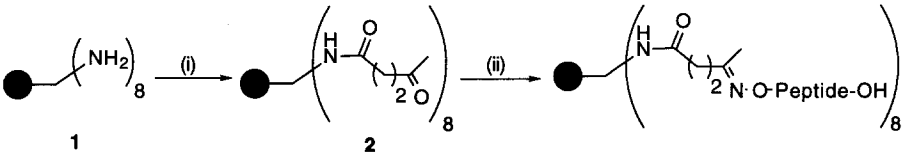
Dendrimeric materials are attracting considerable attention with applications in the areas of molecular recognition, vaccine technology,[1] antiviral agents[2-4] and molecular diagnostics.[5,6] The biological importance of dendritic material has emerged in recent years and there is a need for the development of new and more efficient methods for the synthesis of these materials. It has been found that dendrimeric cores bearing multiple copies of either B- or B- and T-cell epitope peptides are able to elicit strong antibody responses in experimental animals and enhanced sensitivity in solid-phase immunoassays.[1] Furthermore, clustering of carbohydrate ligands upon a multivalent scaffold can provide binding affinities 3-4 orders of magnitude stronger than of the monomeric carbohydrate ligands alone.[7] Thus, sialic acid dendrimers can be used as inhibitors of hemagglutination of human erythrocytes by *Influenza* virus[2,3,8,9] and there is great interest in the discovery of new and more efficient methods for the formation of clustered carbohydrate ligands.[10-12] However, a common feature of all existing methods for the formation of dendrimeric glycoconjugates is the need to engage in significant chemical modification of either the dendrimer core, the carbohydrate modifier, or both.[4,13,14] We sought to develop a new method for the synthesis of a wide variety of biodendrimers using a common strategy. Specifically, we were interested in finding new methods that would allow us to load either peptides or carbohydrates onto an existing dendrimeric scaffold. In this communication, we describe the shortest and most versatile method for the simple synthesis of peptide and carbohydrate dendrimers.

To accomplish this goal, we sought to modify commercially available polyamidoamine (PAMAM) dendrimers in such a way that a simple condensation reaction with an appropriate peptide or carbohydrate would deliver a target biodendrimer in a single step. We were particularly attracted to the oxime-forming condensation reaction between appropriate carbonyl compounds and hydroxylamino nucleophiles, as this chemoselective reaction has proven useful for the formation of a number of different bioconjugates.[15,16] For peptide dendrimers, our strategy was to introduce ketone functionality to the surface of a generation 1 (G1) PAMAM core (**1**) to produce the keto-dendrimer **2** that we then expected would condense with aminooxy peptides to produce a peptide dendrimer (Scheme 1). Our approach to carbohydrate dendrimers involved a polarity reversal: introduction of aminooxy nucleophiles to the surface of G1 PAMAM dendrimers and then oxime-forming condensation with reducing sugars was expected to afford carbohydrate dendrimers.

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00480-1

Synthesis of a G1 PAMAM keto-dendrimer commenced with reaction of commercially available G1 PAMAM with excess levulinic anhydride. Removal of unreacted levulinic acid derivatives and urea byproducts was accomplished by ethyl acetate precipitation of the keto-dendrimer **2** from methanol.[17] Gel permeation and reverse phase HPLC together with MALDI-TOF MS (Calcd 2215; Found 2218.4) and IR analysis of the product provided analytical data consistent with the successful formation of **2**.

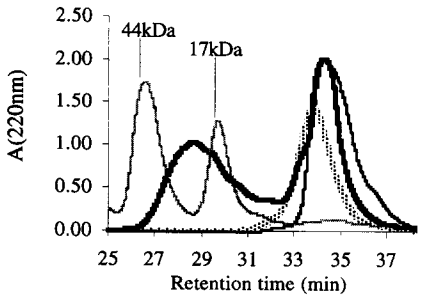
Scheme 1. *Synthesis of peptide dendrimers*[a]



[a]*Reaction conditions:* (i) Levulinic acid (0.4 M), diisopropylcarbodiimide (0.2 M), G1 PAMAM (7 mM) in CH_2Cl_2 then EtOAc precipitation from MeOH; (ii) peptide **3** (2.1 μmol), 8 M urea/0.1 M Na-phosphate buffer (pH 4.7) (6.7 mM), **2** (0.1 μmol, 10 mM), 37 °C, 2 days.

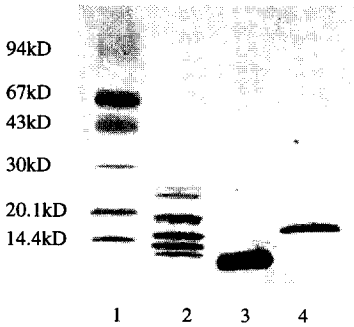
With the keto dendrimer in hand, we prepared a peptide dendrimer by reaction of **2** with a synthetic peptide equipped with an N-terminal aminooxyacetic acid residue. Thus, the peptide (H_2NO-CH_2CO-EYLNKIQNSLSTEWSPASVT-OH, 2.1 μmol, prepared by Chiron Technologies, Melbourne) (**3**) was dissolved in 8 M urea/0.1 M Na-phosphate buffer (pH 4.7) to a concentration of 6.7 mM. This was added to a solution of keto-dendrimer **2** (0.1 μmol, 10 mM) in the same buffer and the solution maintained at 37 °C for 2 days. After this time, gel permeation HPLC showed a new broad peak centered at about 20 kDa as well as unreacted excess peptide (Figure 1). The broad appearance of this new peak indicated that we had successfully prepared dendrimeric peptide derivatives, and that our product consisted of a population of peptide dendrimers with a mean number of five peptides per dendrimer. This was supported by polyacrylamide gel electrophoretic analysis where we observed peptide dendrimers with molecular weights consistent with those expected for dendrimers bearing 8, 7, 6, 5, and 4 copies of peptide (Figure 1). In attempt to fully load the reactive ketones of **2**, we examined the use of *in situ* oxime reduction, warming the reaction mixture for extended periods and resubjection of the partially loaded peptide to fresh batches of peptide. All these attempts failed to enhance the degree of peptide loading and is consistent with the work of others who have attempted to modify existing dendrimer scaffolds.[17] Purification of the peptide dendrimers was accomplished using gel permeation chromatography and allowed us to isolate the peptide dendrimers as well as unreacted peptide.

Figure 1. *Analysis of peptide-PAMAM dendrimers*



GP-HPLC

Dendrimeric Peptide Conjugate (4)
Keto-Dendrimer (2)
Peptide (5)
MW Standards

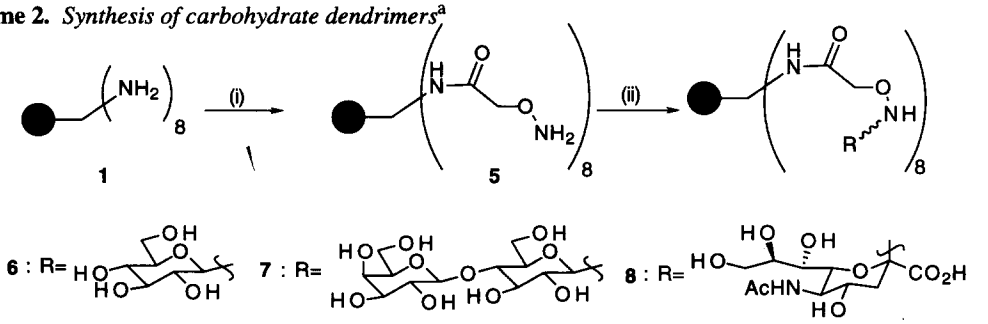


Gel electrophoresis

Lane 1, MW markers; Lane 2 Peptide dendrimers; Lane 3 Unmodified PAMAM (**1**); Lane 4 Lysozyme standard.

We next turned our attention to the synthesis of dendrimeric carbohydrates. It was expected that the nucleophilic aminooxy dendrimer **5** would be capable of intercepting the anomeric equilibrium of suitable carbohydrates via irreversible condensation with their open, carbonyl forms. Synthesis of the G1 PAMAM aminooxy dendrimer was accomplished by reaction of **1** with excess Boc-aminooxyacetic acid (Scheme 2).

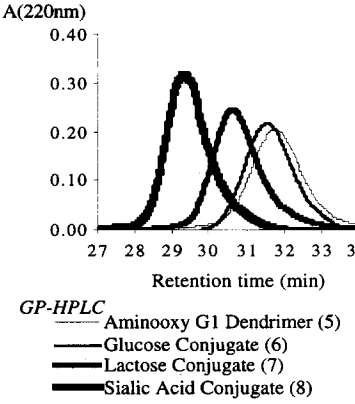
Scheme 2. *Synthesis of carbohydrate dendrimers*[a]

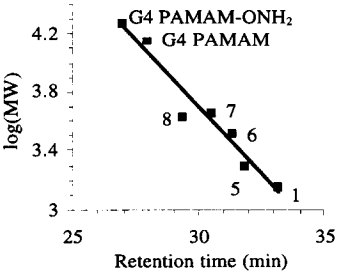


[a] *Reaction conditions:* (i) $BocNHOCH_2CO_2H$ (0.6 M), diisopropylcarbodiimide (0.3 M), G1 PAMAM (10 mM) in CH_2Cl_2:DMF 1:1 then 95%TFA/5% Et_3SiH and Et_2O precipitation from MeOH; (ii) carbohydrate (8.0 μmol), 0.1 M NaOAc (pH 4.0), **5** (0.1 μmol), 2 days.

Deprotection with 95% TFA/5% triethylsilane was followed by precipitation of **5** from methanol using diethyl ether. Both gel permeation HPLC and ESMS (Calcd 2014; Found 2015 $[M+H]^+$) confirmed successful formation of **5**. Glucose, lactose and N-acetylneuraminic acid were chosen as model sugars and each, in turn, was reacted with dendrimer **5**. In a typical procedure, carbohydrate (8.0 μmol) was dissolved in 0.1 M NaOAc buffer (pH 4.0) to a concentration of 0.1 M. This solution was then added to lyophilized aminooxy dendrimer **5** (0.1 μmol), agitated to ensure dissolution of the dendrimer and allowed to stand at ambient temperatures for 2 days. After this time, gel permeation HPLC analysis revealed new peaks of compounds with molecular masses corresponding to those calculated for the gluco- (**6**) and lacto-dendrimer (**7**) respectively (Figure 2).[18] In the case of the sialo-dendrimer (**8**), GP-HPLC again confirmed the formation of a higher molecular weight compound although the observed retention time appeared slightly anomalous.

Figure 2. *Analysis of carbohydrate-PAMAM dendrimers*





Plot of retention time versus log MW for carbohydrate dendrimers

We attribute this behavior to the charged nature of dendrimer **8** that causes secondary polarity effects to become a significant determinant of chromatographic behavior. Figure 2 also shows a plot of retention time versus logMW for dendrimers **5**, **6**, **7**, and **8**. MS analysis of dendrimers **6**, **7**, and **8** using either electrospray or MALDI techniques failed to provide clearly assignable spectra.

In summary, we have shown that the condensation of aminooxy compounds with appropriate aldehydes or ketones serves as a versatile method for the synthesis of both peptide and carbohydrate dendrimers. Our studies now are concerned with the examination of the immunological and diagnostic potential of these dendrimers and the synthesis of higher generation dendrimers using these and related methodologies.

Acknowledgments: We thank Mr. Stuart Thomson for assistance with mass spectral analyses and the Australian Research Council for financial support.

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18. In a control experiment, reaction of unmodified G1 PAMAM (**1**) with lactose also delivered a conjugate, presumably formed by condensation of the dendrimer primary amines with lactose carbonyl groups. However, the formation of this material could be reversed by titration with ethylenediamine. In the case of oxime conjugate **7**, no evidence of reversion to lactose and **5** was observed even upon addition of a 100-fold excess of ethylenediamine