Dendrimers as Carrier Protein Mimetics for IgE Antibody Recognition. Synthesis and Characterization of Densely Penicilloylated Dendrimers

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The synthesis of benzylpenicilloyl-containing dendrimers has been achieved by a convenient procedure involving quantitative functionalization of the terminal amino groups of the three Starbust PAMAM generations used (**G$_n$**; $n = 0, 1, 2$). All these densely penicilloylated dendrimers (**G$_n$P**) exhibit similar, simple NMR spectroscopic data suggesting highly symmetric structures and a monodisperse nature, and the results obtained from MALDI-TOF-MS demonstrate their exact chemical composition. The use of PAMAM dendrimers has allowed us to synthesize, for the first time, carrier benzylpenicilloyl conjugates (**G$_n$P**) of precisely defined chemical structure. The attempts to synthesize **G$_2$P** show that forced experimental conditions are not always useful for the functionalization of the dendrimer, especially in introducing bulky groups. The initial results with sera from patients with different RAST levels were positive and thus suggestive that inhibition occurs, so recognition exists; we can therefore conclude that the hapten−carrier (dendrimer) conjugates studied mimic recognition with natural hapten−carrier (protein) conjugates.

INTRODUCTION

In contrast to linear polymers, dendrimers are highly branched macromolecules with a regular treelike structure of well-defined three-dimensional architecture (*1*). Dendrimers are promising polymeric materials to be used as skeleton building blocks for the development of clusters by virtue of their precisely defined structure (*2*). At the molecular level, dendrimers are potential diagnostic agents thanks to the ease with which they can incorporate multiple functionalities leading to a high activity through multiple interactions that result in increased sensitivity (*3*). Dendritic substances have provided crucial advances; they have already been tested in preclinical studies, particularly as contrast media for magnetic resonance applications (*4*). Dendrimers containing multiple identical ligands are very attractive from the pharmacochemical point of view, since these structures can exhibit amplified substrate binding. Enhanced substrate binding originates either from statistical or cooperativity effects (*5*).

The idea of using a dendritic multifunctional platform to amplify substrate binding has also been exploited to generate antibodies, the improved immunoresponse of which has been ascribed to their high antigen content (*6*). Poly(amidoamine) dendrimers (PAMAM) (*7*) have been used for drug delivery and biomedical applications (*8a*) such as coupling to antibodies to develop immunoassays combining the advantages of hetero- and homogeneous immunoassays (*8b*). The synthesis of several saccharide residues attached to the periphery of preformed PAMAM dendritic cores has been reported, and an increased binding affinity for certain proteins when compared with the individual monomeric sugar unit was demonstrated (*9*).

Current projects in our laboratories are focused on the development of β-lactam hapten−carrier conjugates for quantifying IgE antibodies to penicillins (*10*). Evaluation of subjects with an immediate allergic reaction requires an adequate clinical history plus in vivo and/or in vitro tests (*11*). β-Lactams need to be conjugated with a protein carrier to induce specific IgE antibodies (*12*), and the nature of this molecule is important in the sensitivity of the in vitro test used to detect these antibodies. Since the in vitro tests for detecting specific IgE antibodies to β-lactams are not so sensitive as skin tests, an improvement of in vitro techniques is needed to enhance diagnosis in allergic patients. In this paper we report on the preparation and properties of PAMAM dendrimers functionalized with peripheral benzylpenicilloyl (BPO) units. The benzylpenicilloyl group is the major determinant responsible for the allergic response to β-lactam antibiotics (*13*).

The general aim of this research was to design and synthesize a sequence of precisely defined molecular structures to achieve hapten−carrier conjugates of increasing benzylpenicilloyl density at the periphery of preformed PAMAM dendritic cores and exploiting their exo-receptor properties.

Conventional peptide carrier conjugates, human serum albumin (HSA) or poly-L-lysine (PLL), have the disadvantage of a nonprecise density of haptens in their structure; also, the haptens are randomly distributed in the large protein or peptide carriers. Although tradition-

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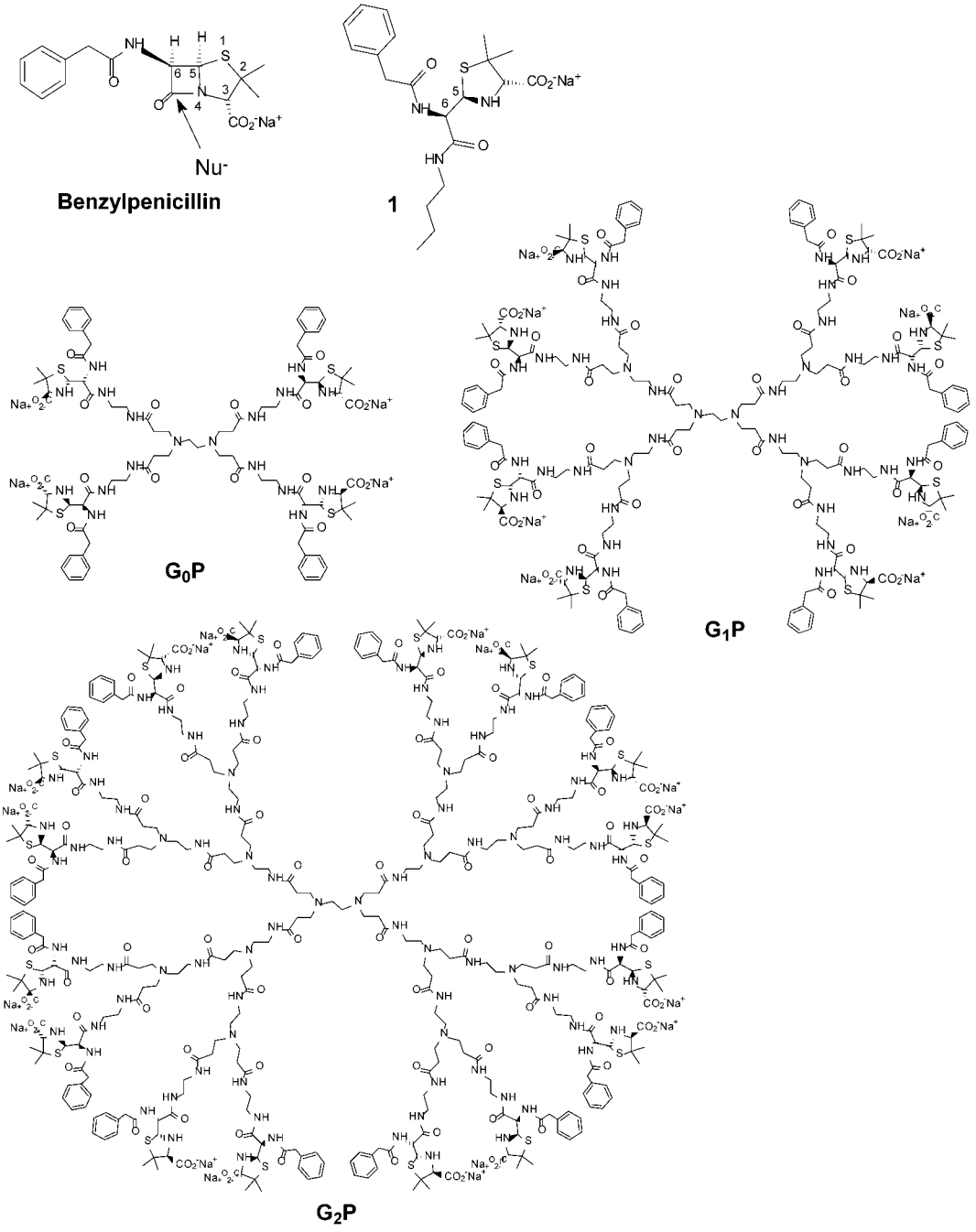
10.1021/bc0155824 CCC: $22.00
Published on Web 04/03/2002

Chart 1. Structures for Model Compound 1 and the Synthesized Dendrimers G_0P, G_1P, and G_2P



ally considered the natural globular carrier, HSA has the limitation of its intrinsic low hapten−carrier density, in addition to the difficulty of establishing the exact structure of its conjugates (*10a*). On the other hand, PLL conjugates—even those with increased hapten−carrier ratios—actually consist of a combination of several structures because PLL is always an average of heterogeneous molecular weight peptides. As a result, this type of conjugate features low reproducibility and reliability in antibody recognition tests (*10b*). Furthermore, the flexibility of polymer chains makes the three-dimensional structures rather variable and difficult to predict. However, the surface and interior of dendrimers are considered segregated (*14*) so all the peripheral haptenic ligands of the conjugates obtained from them should be accessible for binding. Conversely, linear polymers, depending on their conformations, may envelop a substantial fraction of appended ligands.

We chose PAMAM dendrimers due to their availability, generally globular shape and solubility for all molecular sizes. The novel *functional dendritic molecules* (*15*) synthesized from them, reported herein, are part of our program to develop a new in vitro test to quantify *IgE* antibodies to specific β-lactam conjugates with a view to improving the existing methods for diagnosing allergy to this type of antibiotics.

The dendrimers studied, $\mathbf{G_0}$ to $\mathbf{G_2}$, are shown in Chart 1. The chemical properties of the penicilloylated dendrimers were studied and compared with those of the model monomer compound **1** (Chart 1). Covalent coupling of benzylpenicillin to the dendrimers produces novel compounds with unique properties of solubility, high charge density, and specificity. Moreover, these compounds have the ability to remain in an aqueous solution throughout an analytical process (a property considered to be particularly important) (*16*). The structural recogni-

tion of the new compounds from IgE antibodies has been evaluated and compared with that of classical BPO−PLL conjugates.

EXPERIMENTAL PROCEDURES

General. Starburst PAMAM dendrimers (generations zero to two) were purchased from Aldrich as 20 wt % solutions in methyl alcohol. Penicillin G sodium salt was purchased from CEPA, S. L. Standard chemicals were from Aldrich or Merck and used without further purifications. SephadexTM G-10 was purchased from Amersham Pharmacia Biotech AB. The compound **1** was prepared according to the previously reported procedure (*17*). ^{1}H and ^{13}C NMR spectroscopic measurements were carried out on a Bruker WP200 SY or DRX400 BB spectrometer, and chemical shifts (δ) are given in ppm relative to the residual solvent peak. All NMR spectra were recorded using D_2O as solvent (with Na_2CO_3 to ensure a basic pD $\cong$ 11) at room temperature. The MALDI-TOF mass spectra were acquired using a PE Biosystems Voyager System 2081 and were recorded by dissolving the product in 1:1 H_2O/CH_3CN containing 0.1% trifluoroacetic acid (TFA) and using 2,4,6-trihydroxyacetophenone (THAP) (**G_0P** and **G_1P**) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (**G_2P**) as the matrix. The FAB-MS spectrum was obtained using a VG Quattro mass spectrometer and was recorded by using 3-nitrobenzyl alcohol (3-NBA) as the matrix.

General Procedure for the Synthesis of Dendrimers G_nP (n = 0, 1, 2). Penicillin G sodium salt, was added to a stirred solution of the corresponding Starburst PAMAM dendrimer, in 0.02 M aqueous carbonate buffer, pH 10.8, at 20 °C (**G_0P** and **G_1P**) or 4 °C (**G_2P**). The mixture was stirred for the time stated in each case and then purified by gel filtration, using Sephadex G-10 as stationary phase and distilled water as eluent. The solvent was then evaporated in vacuo to obtain the corresponding pure product.

RAST Inhibition Studies. Three sera from patients allergic to penicillins were collected, and specific IgE antibodies were determined by radioallergosorbent tests (RAST) as reported (*17*) using in the solid-phase benzylpenicillin conjugated to PLL (BPO−PLL). Results, expressed as a percentage of maximum label uptake, were considered positive when higher than 2.5%, this being the mean + 2SD of the negative control group (*17*). To study the capacity of the different dendrimers to bind specific IgE antibodies and compare them with the classical monomer used in different studies bound to butylamine **1**, RAST inhibition studies were made as described (*17*). This was carried out using the same solid phase, BPO−PLL. In the fluid phase the sera from patients allergic to penicillin were incubated with three different penicilloylated dendrimers at three 10-fold concentrations, ranging from 25 mM to 0.25 mM for **G_0P**, from 12.5 mM to 0.125 mM for **G_1P**, from 6.25 mM to 0.0625 for **G_2P**, and from 100 mM to 1 mM for **1**. The results were expressed as inhibition percentages.

RESULTS AND DISCUSSION

Penicillins are reactive to a variety of nucleophilic reagents such as alcohols, amines and thiols. The result of this nucleophilic attack is the cleavage of the β-lactam amide bond, which yields penicilloic acid derivatives such as **1**. The rate of reaction of the β-lactam ring in penicillins with amines and other nucleophiles resembles carboxylic acid anhydrides in their acylating capability (*18*). We used this reactivity as the strategy to functionalize the amino terminal groups on the periphery of PAMAM dendrimers. To determine the reaction rate, we previously reacted benzylpenicillin sodium salt with butylamine at NMR scale using D_2O as solvent. The characteristic ^{1}H chemical shifts for protons H-6 and H-5 in the β-lactam ring of benzylpenicillin were used to monitor the progress of the reaction. These two protons resonate at 5.53 and 5.41 ppm ($J_{5,6}$ = 3.9 Hz) in benzylpenicillin, and at 4.85 and 4.30 ppm ($J_{5,6}$ = 8.5 Hz) when the β-lactam ring is open. The reaction was very fast and completed within 15 min. Furthermore, only the product corresponding to the aminolysis of the β-lactam ring was detected (compound **1**). The efficiency and rate of this coupling reaction encouraged us to use the same procedure to synthesize the functionalized dendrimers (**G_nP**).

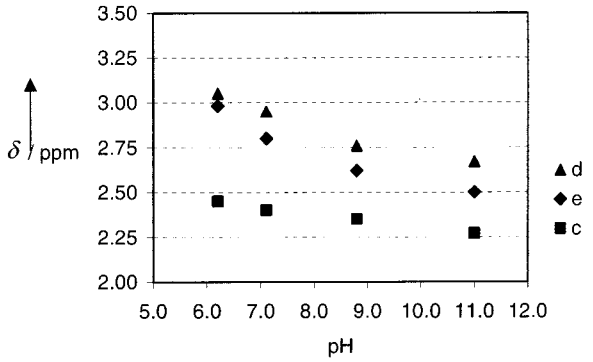


Figure 1. Influence of pH on δ for the inner protons in **G_0P** (for assignments, see Chart 1).

Three different generations of dendrimers were employed (n = 0−2), the reactions being carried out in an aqueous carbonate buffer at pH 10.8 (to ensure that every terminal amino group in the PAMAM dendrimer was deprotonated), using excess benzylpenicillin and controlling the temperature, which turned out to be a strongly influential factor. All products were purified by size-exclusion chromatography and provided essentially pure dendrimers (**G_0P**−**G_2P**) the chemical structure of which was unequivocally assigned by mass spectrometry and ^{13}C and ^{1}H NMR spectroscopy.

The reaction of the zero-generation dendrimer (**G_0**) was monitored by ^{1}H NMR in D_2O, using basic pD obtained by addition of Na_2CO_3. Under these conditions, the ^{1}H NMR spectrum was very clear and contained a single set of signals each of which integrated correctly with the expected number of protons, according to the chemical structure. The resonances for the methylene protons directly bonded to the terminal amino groups were very useful as they clearly reflected deshielding of this signal from 2.70 to 3.22 ppm as the coupling reaction developed. On the other hand, the ^{1}H NMR spectrum recorded in D_2O containing no Na_2CO_3 exhibited two sets of signals that can be assigned to the presence of protonated species (*19*). Therefore, recording the NMR spectra at a basic pD was crucial with a view to evaluating the synthesized dendrimers. Figure 1 shows the influence of pH on the ^{1}H chemical shift (δ) of the inner protons in dendrimer **G_0P**.

The ^{13}C NMR spectrum (Figure 2) was very useful to confirm the structural homogeneity of this compound. While carbons (a) and (b) appeared at 42.78 and 40.82 ppm, respectively, in the zeroth-generation PAMAM dendrimer, they resonated at virtually the same frequency (39.50 and 39.61 ppm) in **G_0P**. The absence of signals for carbons bonded to unsubstituted amino groups confirmed the efficiency of the coupling process.

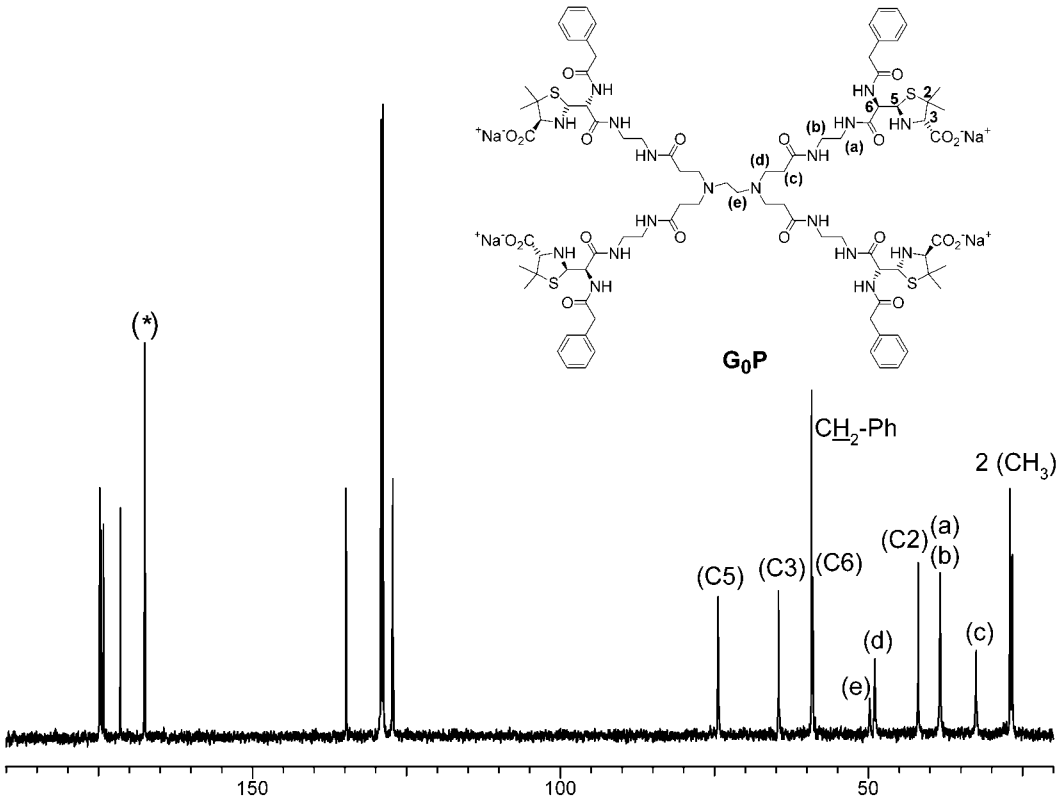


Figure 2. ^{13}C NMR spectrum [100 MHz, D_2O + Na_2CO_3(*)] for dendrimer **G_0P**.

Table 1. Molecular Masses for the Synthesized Dendrimers

		molecular weight	
compound	molecular formula	calculated	observed
G_0P(Na)	$C_{86}H_{116}N_{18}O_{20}S_4Na_4$	1942	1943 $[M+H]^+$ [a]
G_0P(H)	$C_{86}H_{120}N_{18}O_{20}S_4$	1854	1855 $[M+H]^+$ [b]
G_1P(H)	$C_{190}H_{272}N_{42}O_{44}S_8$	4105	4106 $[M+H]^+$ [b]
G_2P(H)	$C_{398}H_{576}N_{90}O_{92}S_{16}$	8606	8607 $[M+H]^+$ [b]

[a] FAB-MS. [b] MALDI-TOF-MS.

Mass spectrometry analysis was carried out using the fast atom bombardment (FAB) and matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) techniques (Table 1). The zeroth-generation dendrimer (**G_0P-(Na)**) was analyzed by FAB-MS, which afforded a molecular ion at $m/z = 1943$ in the spectrum, corresponding to its $[M + H]^+$ adduct. The best conditions for recording the MALDI-TOF mass spectrum for this compound were dissolving the product in 1:1 H_2O/CH_3CN containing 0.1% trifluoroacetic acid (TFA) and using 2,4,6-trihydroxyacetophenone (THAP) as matrix. Under these conditions, the carboxylic moieties in **G_0P** are expected to be protonated (**G_0P(H)**), so a peak corresponding to this structure was found at higher m/z values in the spectrum ($[M + H]^+$).

The first-generation penicilloylated dendrimer (**G_1P**) was synthesized in the same manner, starting from first generation PAMAM dendrimer and using excess benzylpenicillin. The ^1H NMR and ^{13}C NMR spectra were very similar to those for the zeroth-generation dendrimer; the mass spectrometry analysis was performed by using the MALDI-TOF technique under the above-described conditions. A peak matching $[M + H]^+$ for **G_1P(H)** was found in the molecular ion region corresponding to the dendrimer with carboxylic acid moieties in the penicilloyl portions (Table 1).

All attempts at obtaining the functionalized second-generation dendrimer (**G_2P**) under the same conditions as **G_0P** and **G_1P** failed; the result was the second-generation dendrimer with incomplete peripheral substitution. The ^{13}C NMR spectrum showed small signals that could be assigned to carbons bonded to unsubstituted amino groups, as later confirmed by the MALDI-TOF-MS analysis. Attempts involving more drastic conditions (e.g., a higher temperature, an increased concentration of benzylpenicillin, a prolonged reaction time), starting either from the second generation PAMAM dendrimer or the isolated, partially functionalized dendrimer, provided no improvement.

The dendritic structure is a dynamic system (*20*) and, as such, due to the apparent conformational mobility (*21*) it might result in a statistically higher spatial volume engaged with every bulky terminal penicilloyl group. This fact could introduce additional steric hindrance in benzylpenicillin approach to unsubstituted terminal amino groups. However, this restrictive factor could be reduced decreasing the mobility of the partially functionalized dendrimer. Similar observations were reported for a completely different dendrimer family (*9b*) in whitch formation of a "sugar ball" dendrimeric precursor was attempted by terminating the reaction at low temperature.

Bearing in mind this consideration, and the proven efficiency of the coupling process employed, we synthesized **G_2P** at a low temperature (4 °C) to ensure complete peripheral substitution. The penicilloylated dendrimer obtained with this method **G_2P** exhibited ^1H NMR and ^{13}C NMR spectra similar to those for **G_0P** and **G_1P**. The mass spectrometry analysis of the second generation dendrimer, performed using the MALDI-TOF technique and 3,5-dimethoxy-4-hydroxycinnamic acid as matrix, revealed the expected peak at $m/z = 8607$ ($[M + H]^+$,

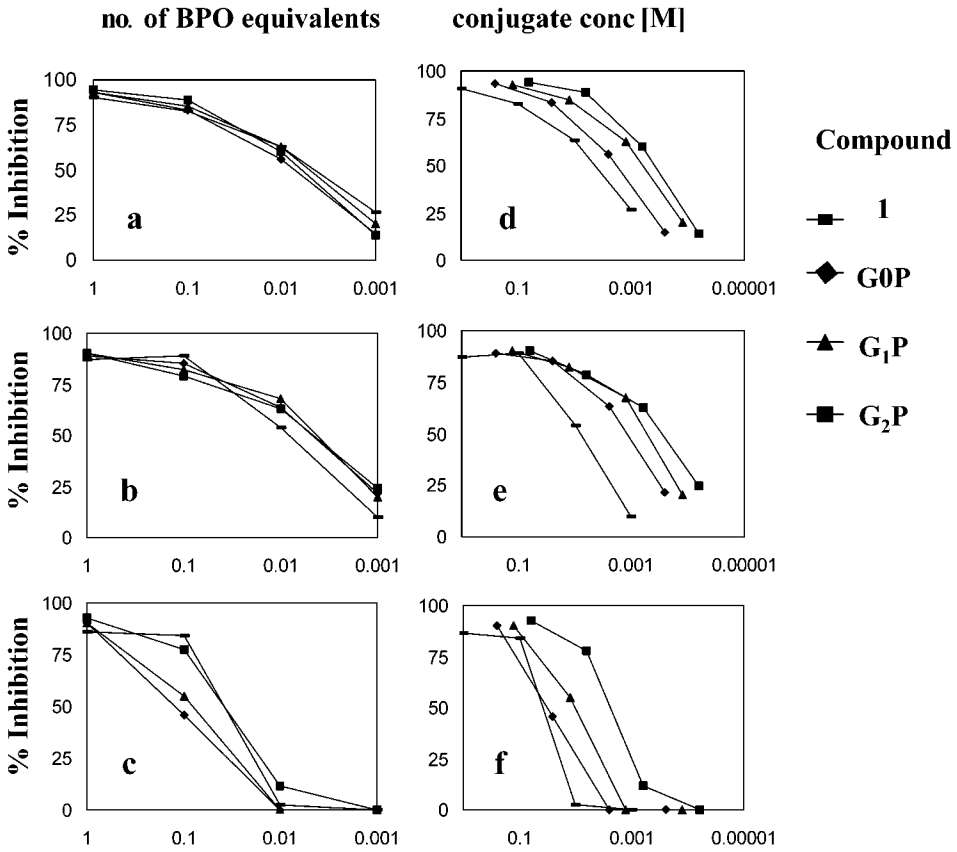


Figure 3. RAST inhibition assay. Sera from patients allergic to penicillins with different specific IgE antibody levels were tested. Specific IgE antibody levels were 7.24 for sera of low affinity (a/d), 9.14 for sera of medium affinity (b/e), and 13.41 for sera of high affinity (c/f). BPO−PLL disks were used in the solid phase. Different dendrimers conjugated to benzylpenicillin **G_0P**, **G_1P**, and **G_2P** and butylamine conjugated to benzylpenicillin **1** were used as inhibitors in the fluid phase.

$G_2P(H)$, Table 1), which confirmed the complete peripheral functionalization of this compound.

To compare the capacity of different dendrimers conjugated to penicillin to bind specific IgE antibodies, RAST inhibition studies were carried out using sera from patients allergic to penicillin. Figure 3 shows the inhibition curves obtained with sera from three patients with different specific IgE antibody levels (ranging from 7 to 13%) determined previously by RAST. As seen, these dendrimers are recognized by the specific IgE antibody from patients allergic to penicillin. We first compared the results obtained with the different conjugates using the same number of penicillin equivalents (Figures 3a, 3b, and 3c). For this, different concentrations of the inhibitors were used, but as they had the same number of penicillin equivalents, there were no differences between the dendrimers and the monomer of butylamine, the pattern being similar in all the sera studied. When we compared these results in terms of conjugate concentrations (Figures 3d, 3e, and 3f), there were clear differences in the capacity of the different dendrimers and butylamine conjugate to bind to specific IgE antibodies. The maximum inhibition in all three cases studied was with **G_2P**, followed by **G_1P**, **G_0P**, and finally **1**. This indicates that a concentration of **1** approximately 100 fold higher than that for **G_2P** is needed to obtain the same percentage inhibition. The differences with the other dendrimers are less clear, although there was a direct correlation between the molecular weight of the dendrimer and the specific IgE antibody binding capacity.

In summary, we synthesized and characterized a series of densely penicilloylated dendrimers (**G_nP**, $n = 0-2$) with precisely defined chemical structures. These dendrimers exhibit very similar, simple ^{1}H NMR and ^{13}C NMR spectra that are suggestive of highly symmetric structures and of the monodisperse nature of these compounds. Furthermore, the mass spectrometry analysis of these functional dendrimers confirmed their exact chemical composition. This constitutes significant progress compared with conventional benzylpenicilloyl conjugates (BPO−HSA and BPO−PLL).

The proposed approach to the synthesis of dendrimer **G_2P** can be a useful tool for the functionalizing of this type of dendrimer provided the coupling process employed is efficient enough. As noted earlier, the use of "forcing conditions" led to no improved results.

Preliminary RAST inhibition studies have confirmed the usefulness of penicilloylated dendrimers as conjugates for the recognition of IgE antibodies from patients allergic to penicillin. We are now ready to evaluate the relevance of the size of the hapten−carrier conjugate and hapten density profile in recognition, simply by changing the generation of the starting dendrimer (in our study there was an increase in the percent RAST inhibition from generation 0 to 2). Thus, presenting many penicillin determinants to specific IgE antibodies using structures able to carry many hapten molecules would increase in vitro sensitivity. Consequently, these compounds can be considered promising candidates for use in the development of a new in vitro test, with increased sensitivity for the diagnosis of allergy to β-lactam antibiotics compared to the test used until now. Work is in progress in our laboratories.

ACKNOWLEDGMENT

This work was supported by funds granted for the realization of the FEDER Project 1FD97-0516. F.S-S is grateful for the award of a fellowship related to this project. The authors are also grateful to Prof. E. Giralt (Departamento de Química Orgánica, Universidad de Barcelona, Spain) for recording the MALDI-TOF-MS spectra and to Dr. M. Maestro (SXAIN, Universidad de La Coruña, Spain) for the FAB-MS spectrum.

Supporting Information Available: Preparation procedure, chemical structure, ^{1}H and ^{13}C NMR data, and MALDI-TOF-MS data and spectra for **G_0P**, **G_1P**, and **G_2P**; FAB-MS data for **G_0P**. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC0155824