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Research Article

Synthesis of PAMAM Dendrimers Loaded with Mycophenolic Acid to Be Studied as New Potential Immunosuppressants

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The terminal *N*-Boc protected diamino PAMAM **7** was condensed (EDC-DMAP) with two units of mycophenolic acid (MPA) giving the *N*-Boc protected dendron **8** in a good yield (76%). The ammonium trifluoroacetate **9** was prepared from **8** by acid treatment (TFA-THF-H_2O) and was split into two equal parts. The first half was treated with di-2-pyridyl thionocarbonate (DPT) in the presence of Et_3N to give the corresponding isothiocyanate **10**. This was reacted with the second half of **9** providing the symmetrical dendrimer **11** (68% yield), exposing four MPA units around the thioureido-PAMAM core.

1. Introduction

Mycophenolic acid (MPA, **1**) is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the *de novo* synthesis of guanine nucleotide [1]. MPA exhibits antiproliferation activity and has been established as an anticancer agent, an immunosuppressant [1, 2] as well as an antiviral drug against some plant viruses [3–5]. Among these promising properties of MPA, the immunosuppressant property is by far the most important. MPA is a cornerstone immunosuppressant used to prevent rejection after organ transplantation. Currently, two MPA derivatives are used clinically for this purpose (Figure 1): mycophenolate sodium (MPS, Myfortic, **2**) and mycophenolate mofetil (MMF, CellCept, **3**). Several structural modification of MPA were carried out during the last twenty years to advance the elucidation of the structure-activity relationship of MPA in its interaction with IMPDH and contributed to the understanding of the relevant sites required for drug activity. Analogues which displayed similar or better immunosuppressive activity were reported. These derivatives present modifications only at the terminal carboxylic acid [6–11].

In the search of new immunosuppressants, we planned the preparation of a new family of MPA derivatives linking more units of MPA to a scaffold by means of the carboxylic group. Among the possible scaffolds, we decided to use dendrimers as a drug delivery system (**4**, Figure 1) [12]. These are nanostructured macromolecules characterized by a tree-like architecture with exponential numbers of discrete dendritic branches radiating out from a common core. One of the most commonly known dendrimeric structure is the poly(amidoamine) (PAMAM) dendrimer which was introduced and developed by Tomalia and Fréchet [13]. PAMAM dendrimers are monodispersed, biocompatible, and synthetic macromolecules with well-defined structural architecture and composition. Here, we present the synthesis of PAMAM derivatives decorated with MPA units. The antiviral activity of these compounds has been recently tested in *Nicotiana tabacum* L. cv. Xanthi explants infected with a cucumber mosaic virus [14].

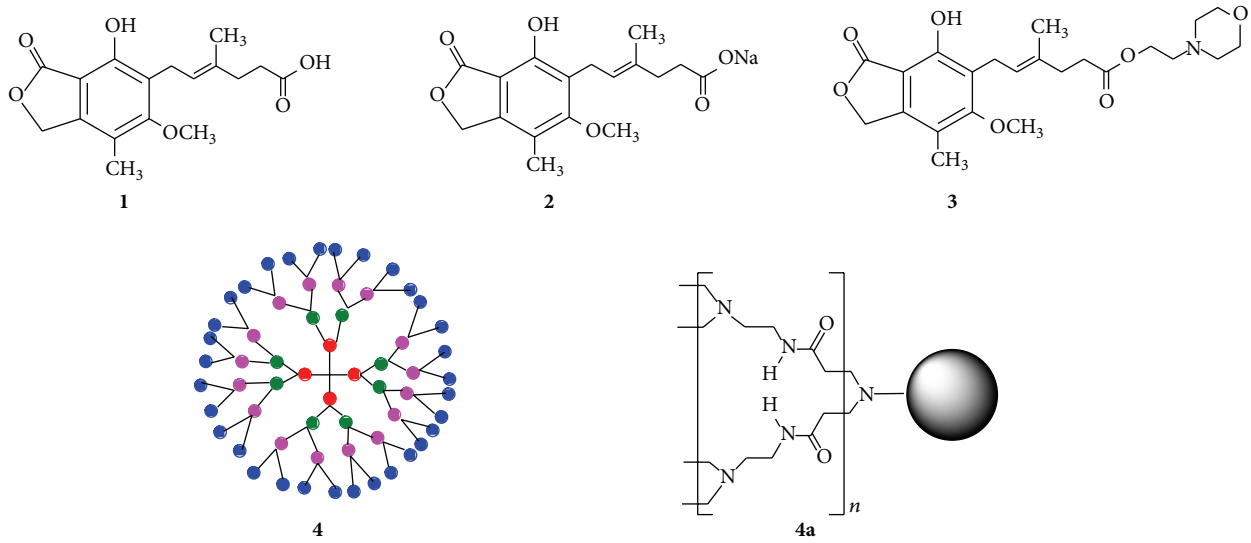


FIGURE 1: Mycophenolic acid (**1**) and its marketed derivatives (**2**) and (**3**); schematic representation of the structure of a generic dendrimer (**4**) and of a single PAMAM branch (**4a**).

2. Experimental

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 ± 2°C. ^{1}H NMR and ^{13}C NMR spectra were recorded in appropriate solvents with a Bruker Avance II spectrometer operating at 250.12 MHz (^{1}H) and 62.9 MHz (^{13}C). Spin resonances were reported as chemical shifts (δ) in part per million (ppm) and the references to the residual peak of the solvent employed as follows: CD_3CN 1.94 ppm (^{1}H NMR, central band) and 1.28 ppm (^{13}C NMR, central band), CD_3OD 3.31 ppm (^{1}H NMR, central band) and 49.0 ppm (^{13}C NMR, central band). Spin multiplicity was indicated by s = singlet, d = doublet, t = triplet, bt = broad triplet, q = quartet, m = multiplet. Coupling constants J were reported in Hertz (Hz). The assignments were made, when possible, with the aid of DEPT, HETCOR, COSY, and HSQC experiments. Low resolution mass spectra were recorded on a LCQ Advantage ThermoFinnigan spectrometer equipped with an ion trap analyzer (Thermo Electron Company, San Jose, CA, USA). All reactions were followed by TLC on Kieselgel 60 F_{254} with detection by UV light and/or with ethanolic 10% phosphomolybdic or sulfuric acid and heating. Kieselgel 60 (E. Merck, 70–230 and 230–400 mesh, resp.) was used for column and flash chromatography. Solvents were dried and purified by distillation according to standard procedure [15] and stored over 4 Å molecular sieves activated for at least 24 h at 200°C. $MgSO_4$ was used as the drying agent for solutions. Compounds **5** and **7** were prepared according to the reported procedures [16].

2.1. Synthesis of Compound ***6****.* A solution of mycophenolic acid (**1**) (125 mg, 0.390 mmol, 1.2 eq) and amine **5** (57 mg, 0.354 mmol) in dry DMF (1.5 mL) containing Et_3N (148 μL, 1.06 mmol, 3.0 eq) was treated at room temperature with 1-ethyl-3-(3-(dimethylamino)-propyl)-carbodiimide hydrochloride (EDC, 90.4 mg, 0.472 mmol, 1.34 eq) and *N*-hydroxybenzotriazole (HOBt, 49.2 mg, 0.364 mmol, 1.03 eq). The solution was stirred at room temperature until TLC analysis (9 : 1 CH_2Cl_2-MeOH, 21 h) revealed the complete disappearance of amine **5** and the formation of a major product (R_f = 0.85). The mixture was concentrated under diminished pressure and the purification of the residue by flash chromatography over silica gel (2 : 8 hexane-EtOAc) gave pure **6** (120 mg, 73% calculated from **5**) as a solid foam; R_f = 0.22 (2 : 8 hexane-EtOAc); ^{1}H NMR (250.12 MHz, CD_3CN-$CDCl_3$): δ 1.38 [s, 9H, C(CH_3)$_3$], 1.92 (s, 3H, =CCH_3), 2.10 (s, 3H, Ar-CH_3), 2.18–2.24 (m, 4H, =C(CH_2)$_2$CO), 2.98 (q, 2H, J = 5.7 Hz, CH_2NHCO), 3.10 (q, 2H, J = 5.7 Hz, CH_2NHCOO), 3.34 (d, 2H, J = 7.0 Hz, ArCH_2CH=), 3.73 (s, 3H, CH_3), 5.20 (s, 2H, ArCH_2OCO), 5.17 (t, 1H, J = 7.0 Hz, CH=C), 5.42 (bt, 1H, NHCO), 6.53 (bt, 1H, NHCOO), 7.74 (s, 1H, OH); ^{13}C NMR (62.9 MHz, CD_3CN-$CDCl_3$): δ 11.6 (Ar-CH_3), 16.3 (=CCH_3), 23.1 (ArCH_2CH=), 28.5 [C(CH_3)$_3$], 35.4, 35.8 (=C(CH_2)$_2$CO), 40.0, 40.9 [HN(CH_2)$_2$NH], 61.5 (OCH_3), 70.8 (ArCH_2O), 79.3 [C(CH$_3$)$_3$], 107.2 (C=), 123.3 (CH=), 116.0, 122.5, 135.3, 145.8 (4 × Ar-C), 157.0 (O-C=O), 153.8, 164.2 (2 × Ar-C-O), 173.2, 173.6 (2 × C=O). ESIMS: calcd for $C_{24}H_{34}N_2O_7Na$ $[M + Na]^+$ 485.5, found 485.3. Anal. Calcd for $C_{24}H_{34}N_2O_7$: C, 62.32; H, 7.41; N, 6.06. Found: C, 62.34; H, 7.44; N, 6.08.

2.2. Synthesis of Compound ***8***

Method A (EDC, HOBT, Et_3N, and DMF). A solution of mycophenolic acid (**1**) (369 mg, 1.151 mmol, 1.2 eq) and amine **7** (204 mg, 0.525 mmol) in dry DMF (6.0 mL) containing Et_3N (48 μL, 3.48 mmol, 3.0 eq) was treated at room temperature with EDC (296 mg, 0.514 mmol, 1.33 eq) and HOBt (161 mg, 1.19 mmol, 1.03 eq) and the solution was stirred at

room temp. After 48 h, TLC analysis (8 : 2 CH_2Cl_2-MeOH) revealed the complete disappearance of amine **7** and the formation of a major product (R_f = 0.71). The mixture was concentrated under diminished pressure and the purification of the residue by flash chromatography over silica gel (88 : 12 CH_2Cl_2-MeOH) gave pure **8** (298 mg, 57% calculated from **7**).

Method B (EDC, DMAP, and DMF). A solution of amine **7** (98.4 mg, 0.253 mmol) and mycophenolic acid (**1**) (179 mg, 0.558 mmol, 2.2 eq) in dry DMF (3 mL) was treated with EDC (130 mg, 0.676 mmol, 2.67 eq) and 4-dimethylaminopyridine (DMAP, 9.3 mg, 0.0759 mmol, 0.3 eq). The mixture was stirred at room temperature and after 48 h the TLC analysis (9 : 1 CH_2Cl_2-MeOH) revealed the complete disappearance of amine **7** and the formation of a major product (R_f = 0.40). The mixture was concentrated under diminished pressure and the purification of the residue by flash chromatography over silica gel (CH_2Cl_2-MeOH 92 : 8) gave pure **8** (191 mg, 76%).

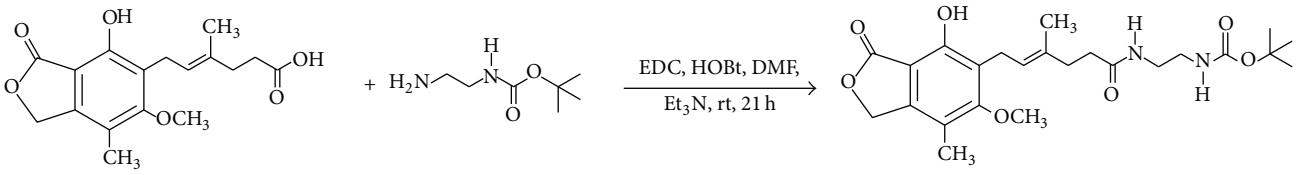
Compound **8** was a solid foam; R_f = 0.12 (92 : 8 CH_2Cl_2-MeOH); ^{1}H NMR (250.12 MHz, CD_3OD-$CDCl_3$): δ 1.40 [s, 9H, C(CH_3)$_3$], 1.80 (s, 6H, 2 × =CCH_3), 2.13 (s, 6H, 2 × Ar-CH_3), 2.30 (bt, 4H, J = 6.3 Hz, 2 × NCH_2CH_2CONH), 2.32 (bs, 8H, 2 × =C$(CH_2)_2$CO), 2.50 (t, 2H, J = 6.8 Hz, NCH_2CH_2NHCOO), 2.75 (bt, 4H, J = 6.3 Hz, 2 × NCH_2CH_2CONH), 3.15 (t, 2H, J = 6.8 Hz, NCH_2CH_2NHCOO), 3.30 [m, 8H, 2 × $CONH(CH_2)_2NHCO$], 3.36 (d, 4H, J = 6.6 Hz, 2 × ArCH_2CH=), 3.75 (s, 6H, 2 × OCH_3), 5.41 (m, 6H, 2 × CH=C, 2 × ArCH_2OCO); ^{13}C NMR (62.9 MHz, CD_3OD-$CDCl_3$): δ 11.6 (2 × ArCH_3), 23.5 (2 × ArCH_2CH=), 16.3 (2 × =CCH_3), 33.4 (2 × NCH_2CH_2CONH), 28.7 [C(CH_3)$_3$], 35.6, 36.3 (2 × =C$(CH_2)_2$CO), 39.8, 39.7 [2 × HN$(CH_2)_2$NH], 38.4 (NCH_2CH_2NHCOO), 50.8 (2 × NCH_2CH_2CONH), 53.1 (NCH_2CH_2NHCOO), 61.5 (2 × OCH_3), 70.7 (2 × ArCH_2O), 80.1 [C(CH_3)$_3$], 107.5 (2 × C=), 123.9 (2 × CH=), 117.7, 123.3, 134.8, 145.8 (8 × Ar-C), 158.1 (O-C=O), 154.5, 164.6 (4 × Ar-C-O), 173.6, 174.3, 175.6 (6 × C=O). ESIMS: calcd for $C_{51}H_{72}N_2O_{14}Na$ [M + Na]$^+$ 1015.5, found 1015.4. Anal. Calcd for $C_{51}H_{72}N_6O_{14}$: C, 61.68; H, 7.31; N, 8.46. Found: C, 61.70; H, 7.33; N, 8.47.

*2.3. Synthesis of Dendrimer **11**.* A solution of **8** (186 mg, 0.186 mmol) in CH_2Cl_2-TFA-H_2O 7.4 : 2.4 : 0.2 (2.0 mL) was stirred at room temperature until the TLC analysis (8 : 2 CH_2Cl_2-MeOH, 1 h) revealed the complete disappearance of the starting material and the formation of a major product (R_f = 0.36). The reaction mixture was concentrated under diminished pressure and repeatedly coevaporated with toluene (4 × 30 mL). The crude residue (184 mg) analyzed by NMR proved to be constituted only by the ammonium trifluoroacetate **9**. The salt **9** is a solid foam, R_f = 0.36 (8 : 2 CH_2Cl_2-MeOH); ^{1}H NMR (250.12 MHz, CD_3CN): δ 1.77 (s, 6H, 2 × =CCH_3), 2.10 (s, 6H, 2 × Ar-CH_3), 2.70 (bs, 4H, 2 × NCH_2CH_2CONH), 2.18–2.30 (m, 12H, 2 × =C$(CH_2)_2$CO, 2 × NCH_2CH_2CONH), 3.05 (m, 2H, $NCH_2CH_2NH_3^+$), 3.32 (d, 4H, J = 6.8 Hz, 2 × ArCH_2CH=), 3.20, 3.40 [2m, each 4H, 2 × $CONH(CH_2)_2NHCO$], 3.44 (m, 2H, $CH_2NH_3^+$), 3.72 (s, 6H, 2 × OCH_3), 5.16 (m, 6H, 2 × CH=C, 2 × ArCH_2OCO), 10.1 (bs, 4H, 4 × NHCO), 7.80 (s, 2H, 2 × OH); ^{13}C NMR (62.9 MHz, CD_3CN): δ 11.6 (2 × Ar-CH_3), 16.2 (2 × =CCH_3), 23.3 (2 × ArCH_2CH=), 29.1 (2 × NCH_2CH_2CONH), 35.9, 36.4 (2 × =C$(CH_2)_2$CO), 38.7, 39.9 [2 × HN$(CH_2)_2$NH], 51.9 (2 × NCH_2CH_2CONH), 36.4 (CH_2NHCO), 50.1 ($CH_2NH_3^+$), 61.6 (2 × OCH_3), 71.0 (2 × ArCH_2O), 123.8 (2 × CH=), 107.3 (2 × C=), 119.0, 122.7, 135.2, 146.2 (8 × Ar-C), 153.9, 164.3 (4 × Ar-CO), 173.0, 176.0, 173.4 (6 × C=O).

To a solution of crude **9** (92 mg, 0.0912 mmol) in dry CH_2Cl_2 (1.4 mL), Et_3N (100 μL) and 2-pyridyl thiocarbonate (DPT, 22 mg, 0.0912 mmol) were added at room temperature. After 6 h the TLC analysis (7 : 3 EtOAc-^{i}prOH) revealed the complete disappearance of the starting material and the formation of a major product (R_f = 0.24). The solution was concentrated under diminished pressure and the crude isothiocyanate intermediate **10** (86 mg, 0.0912 mmol) was solubilized in dry 1 : 1 CH_2Cl_2-DMF (1.0 mL) and a solution of crude **9** (92 mg, 0.0912 mmol) in dry 1 : 1 CH_2Cl_2-DMF (2.0 mL) containing Et_3N (0.2 mL) was added dropwise. The reaction mixture was stirred at room temperature for 43 h, until TLC analysis (7 : 3 EtOAc-^{i}prOH) revealed the formation of a major product at R_f = 0.22. The reaction was concentrated at reduced pressure and the crude was subjected to a flash chromatography (EtOAc) on silica gel to give dendrimer **11** (114 mg, 68% from **9**) as a foam solid; R_f = 0.22 (7 : 3 EtOAc-^{i}prOH); ^{1}H NMR (250.12 MHz, CD_3OD-$CDCl_3$): δ 1.81 (s, 12H, 4 × =CCH_3), 2.14 (s, 12H, 4 × Ar-CH_3), 2.26 [bs, 16H, 4 × =C$(CH_2)_2$CO], 2.34 (bt, 8H, J = 6.4 Hz, 4 × NCH_2CH_2CONH), 2.72–2.86 (m, 12H, 2 × NCH_2CH_2NHCS, 4 × NCH_2CH_2CONH), 3.22 [m, 16H, 4 × $CONH(CH_2)_2NHCO$], 3.37 (d, 8H, J = 7.0 Hz, 4 × ArCH_2CH=), 3.58 (t, 4H, J = 5.6 Hz, NCH_2CH_2NHCS), 3.76 (s, 12H, 4 × OCH_3), 5.21 (s, 8H, 4 × ArCH_2OCO), 5.24 (t, 4H, J = 7.3 Hz, 4×CH=C); ^{13}C NMR (62.9 MHz, CD_3CN-D_2O): δ 11.5 (4 × Ar-CH_3). 16.3 (4 × =CCH_3), 23.5 (4 × ArCH_2CH=), 34.8 (4 × NCH_2CH_2CONH), 35.8, 36.4 [4 × =C$(CH_2)_2$CO], 39.9 [4 × HN$(CH_2)_2$NH], 44.3 (NCH_2CH_2NHCS), 50.8 (4 × NCH_2CH_2CONH), 54.2 (2 × NCH_2CH_2NHCS), 61.5 (4 × OCH_3), 70.7 (4 × ArCH_2O), 107.5 (4 × C=), 117.6, 123.4, 134.9, 146.2 (16 × Ar-C), 124.3 (4 × CH=), 154.5, 164.5 (8 × Ar-C-O), 173.6, 174.7, 175.7 (12 × C=O), 179.8 (C=S). ESIMS: calcd for $C_{93}H_{126}N_{12}O_{24}SNa$ [M + Na]$^+$ 1849.9, found 1849.7. Anal. Calcd for $C_{93}H_{126}N_{12}O_{24}S$: C, 61.10; H, 6.95; N, 9.19. Found: C, 61.13; H, 6.98; N, 9.21.

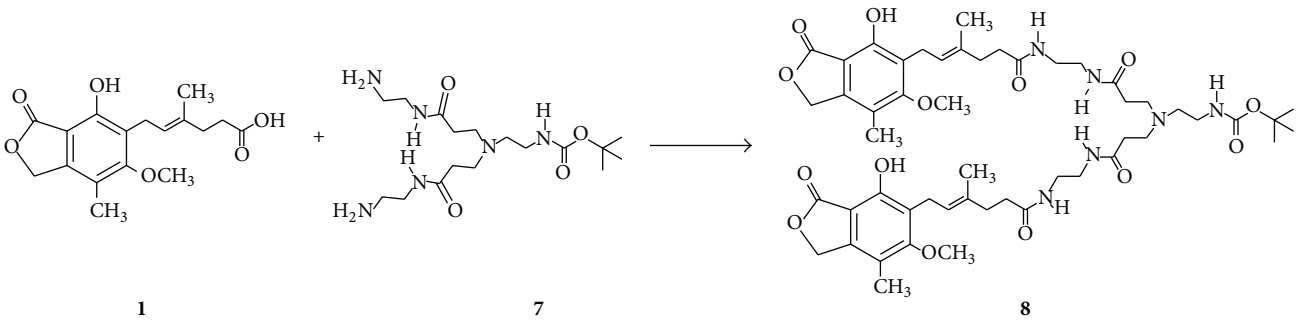
3. Results and Discussion

Derivative **6** was first prepared, as a positive control for biological evaluations (Scheme 1). The monoprotected *N*-Boc-ethylenediamine **5** [16], which is also used as the central core in the construction of PAMAM dendrons, was used as capping reagent. The same kind of additional functional groups will be present in all synthetic structures, thus ensuring similar interactions during the evaluation of immunosuppressant properties.



1 **5** **6** (73% yield)

Scheme 1: Synthesis of compound **6**.



1 **7** **8**

Scheme 2: Synthesis of compound **8**. Reaction conditions: EDC, DMPA, DMF, room temperature, 48 h (76% yield) or EDC, HOBt, DMF, Et_3N, room temperature, 48 h (57% yield).

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which is nowadays preferred to *N*,*N*′-dicyclohexylcarbodiimide (DCC) in the formation of amide bonds because it is not a skin sensitizer and its urea by-product can be easily removed, was chosen as a coupling agent and the reaction was carried out at room temperature in the presence of *N*-hydroxybenzotriazole (HOBt) and triethylamine (Et_3N). Compound **6** was isolated after 12 h in a 73% yield after flash chromatography on silica gel. The two-branched structure **7** [16] was easily obtained via double Michael addition of methyl acrylate to *N*-Boc-ethylenediamine followed by amidation of the intermediate with a large excess of ethylenediamine. This was then reacted with **1** using the same coupling conditions seen above (Scheme 2). The reaction required longer time (48 h) to go to completion and afforded **8** in a poor 57% yield. Better results were obtained when *N*,*N*-dimethylaminopyridine (DMAP) was used instead of the mixture Et_3N-HOBt. In this case, the desired compound was isolated in a 76% yield after chromatographic purification.

Having obtained this structure, the synthesis of the symmetrical dendrimer **11** was possible (Scheme 3). First, the acid labile *N*-Boc protecting group was removed (trifluoroacetic acid-water, in CH_2Cl_2) giving the ammonium trifluoroacetate salt **9** in a quantitative yield. The structure and purity of **9** were confirmed by NMR analysis, which verified the complete removal of the *t*-butoxy carbonyl group. The latter compound was then reacted, without purification, with di-2-pyridyl thionocarbonate (DPT) and triethylamine. DPT [17, 18] is a commercially available, solid, nontoxic reagent that can be used in the preparation of isothiocyanates as a safe alternative to thiophosgene. The reaction proceeded smoothly and gave the complete conversion after 5.5 h. The intermediate isothiocyanate obtained after removal of the solvents was directly reacted with crude **9** in the presence of Et_3N in a mixture of CH_2Cl_2 and DMF.

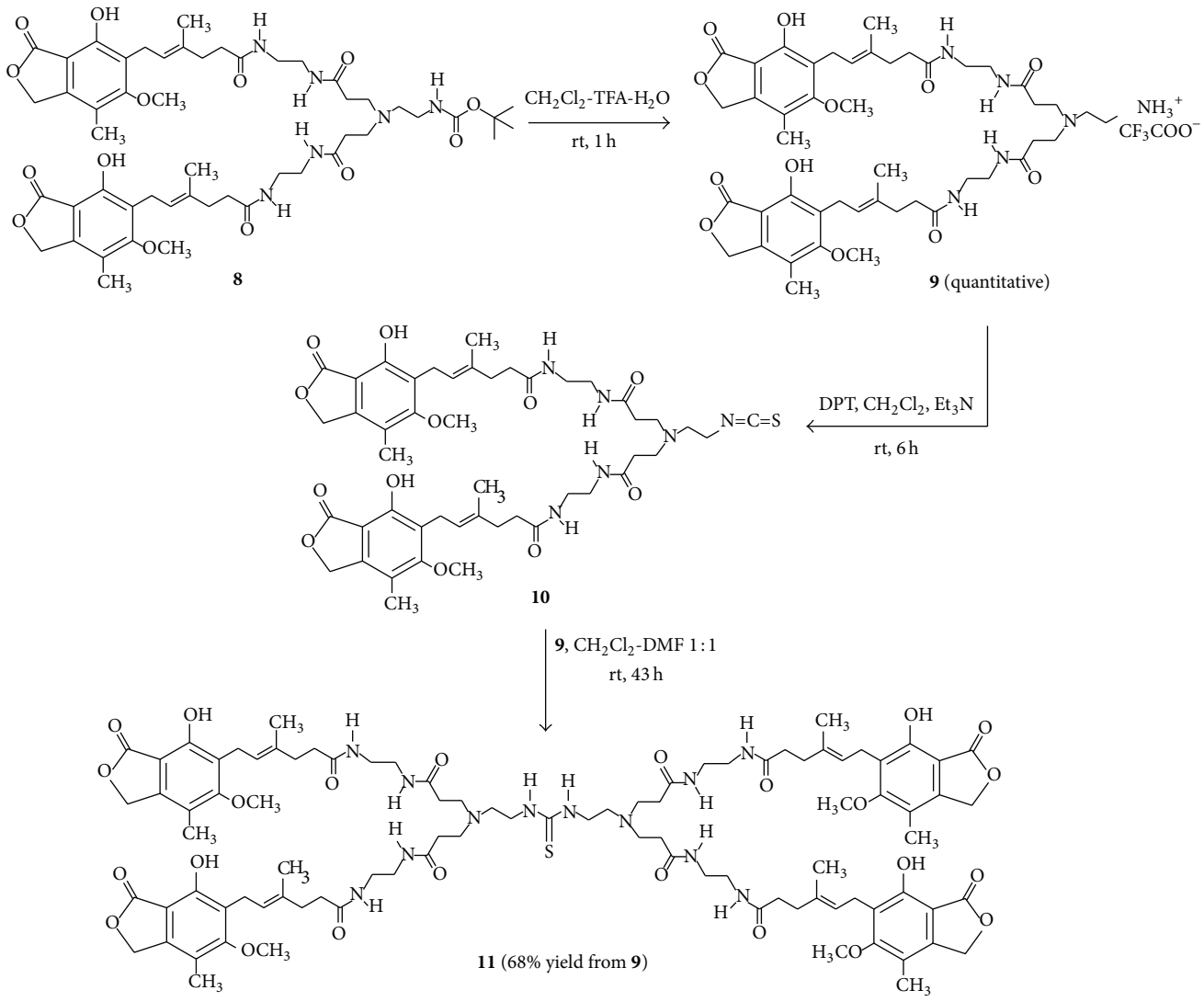
Dendrimer **11** was obtained after flash chromatographic purification in a satisfactory 68% overall yield. The symmetrical structure of compound **11** and the formation of the thioureido bridge (diagnostic C=S signal) were fully confirmed by ^1H, ^{13}C and 2D NMR experiments.

4. Conclusions

In conclusion, we reported the synthesis of poly (amidoamine) structures decorated with mycophenolic acid. The target structures were prepared in good overall yields and with a limited number of purification steps. Furthermore, the very same strategy could be used to prepare dendrimers of higher generation. On the other hand, the presence of the orthogonally protected amino group on the inner core could allow for the preparation of a library of asymmetrical structures coupling the dendron with different compounds rather than its symmetrical counterpart. The immunosuppressant properties of these compounds are currently under investigation and the results will be published in due course.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.



Scheme 3: Synthesis of dendrimer **11**.

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