Bioorganic & Medicinal Chemistry Letters 20 (2010) 1869–1872

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel powerful water-soluble lipid immunoadjuvants inducing mouse dendritic cell maturation and B cell proliferation using TLR2 pathway

Maria Vittoria Spanedda [a], Béatrice Heurtault [a], Steffen Weidner [a], Corinne Baehr [a], Emmanuelle Boeglin [b], Julien Beyrath [b], Sara Milosevic [b], Line Bourel-Bonnet [a], Sylvie Fournel [b,*], Benoît Frisch [a,*]

[a] Equipe de Biovectorologie, Laboratoire de Conception et Application des Molécules Bioactives, UMR 7199 CNRS/Université de Strasbourg, Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch Cedex, France
[b] Immunologie et Chimie Thérapeutiques, UPR 9021 CNRS, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France

ARTICLE INFO

Article history:
Received 14 December 2009
Revised 28 January 2010
Accepted 29 January 2010
Available online 2 February 2010

Keywords:
TLR2 agonist
Water-soluble
Immunoadjuvant

ABSTRACT

Four novel water-soluble lipid immunoadjuvants were designed, synthesized and characterized by MS and NMR. They all induce mouse dendritic cell maturation and B cell proliferation. We demonstrate that in spite of the chemical modification, the four compounds remain TLR2 agonists.

In the present vaccine context, new original immunoadjuvants are more than ever needed.[1–3] In this field, synthetic analogs of triacylated and diacylated lipopeptides derived from the N-terminal domain of respectively bacterial or mycoplasmal lipoproteins are highly potent immunoadjuvants when administered either in combination with protein antigens or covalently linked to small antigenic peptides.[4,5] It is now well established that these molecules act as agonists on Toll Like Receptors (TLRs) heterodimers and activate cells of innate and adaptive immune systems as agonists. They act specially on TLRs 2/1 and 2/6 (for triacylated and diacylated lipopeptides, respectively).[6,7]

Despite little is known about the molecular basis of ligand recognition, one of the key physico-chemical properties of this kind of compounds is their amphiphilicity. Though it is a cornerstone in their activity, amphiphilicity settles some practical problems of solubility that are overcome when the above-mentioned lipoadjuvants are covalently bound to hydrophilic proteins or peptides imposing their hydrophilicity on the final product. Amphiphilicity is also turned as an advantage in vaccine experiments where, for example, an analogue of 'Pam$_3$CAG' (in other words, *N*-α-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycine, **1**) is incorporated into liposomes. The fatty part of these synthetic lipopeptides interacts with liposome lipid bilayer whereas their hydrophilic head is modified covalently to anchor one or two peptide containing T epitopes against which an immune response is expected.[8,9] Nevertheless, if such a formulation is not desirable, the amphiphilicity is a drawback that hampers the easy use of lipopeptidic immunoadjuvants both in vitro and in vivo. Several studies proved that lipopeptides aggregated in water solutions and that large and heterogeneous aggregates were responsible for the loss of activity.[10] In this context, it has been reported that activity of such lipopeptides was critically dependent on the dilution protocol (e.g., with dimethylsulfoxide or *tert*-butyl alcohol) and on the presence of proteins (fetal bovine serum, bovine serum albumin...) or detergents (octyl-β-D-glucopyranoside) acting as solubilizers.[11]

To avoid complicated dissolution/dispersion protocols and the use of solubilizers, we developed synthetic analogs of 'Pam$_3$CAG' (**1**) that could be more hydrophilic and, if possible, water-soluble. To increase the water solubility of compounds of interest, various strategies could be used[12] as, for example, the introduction of supplementary charges on the molecule. For this, the addition of protonable functions on lipopeptidic immunoadjuvant thanks to a

Abbreviations: Boc, tertiobutyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; CD, cluster of differentiation; DC, dendritic cell; DCC, dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; DMAP, dimethylaminopyridine; LPS, lipopolysaccharide; Pam, palmitoyl; Pam$_3$CAG-OH, *N*-α-palmitoyl-*S*-[23-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteinyl-L-alanyl-glycine; PEG, polyethyleneglycol; TFA, trifluoroacetic acid; TLR, toll-like receptor.

* Corresponding authors. Tel.: +33 (0)3 88 41 70 24; fax: +33 (0)3 88 61 06 80 (S.F.); tel.: +33 (0)3 68 85 41 68; fax: +33 (0)3 68 85 43 06 (B.F.).
E-mail addresses: s.fournel@unistra.fr (S. Fournel), frisch@unistra.fr, lbourel@unistra.fr (B. Frisch).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.01.146

polylysine head has already been described and is commercially available.[13] Nevertheless, such an addition reinforces the amphiphilic character of the analogs, therefore non-specific adhering properties can be encountered. To avoid the polycationic disturbance, we chose to increase the overall hydrophilicity without modifying the final charges. Therefore, we chose to add a polyethylene glycol (PEG) chain.[14] A lipopeptide-PEG conjugate was previously evaluated on the lymphocyte B activity and was found to keep the same properties than the hydrophilic counterpart.[15] Moreover, Heucking and co-workers recently reported the characterization of similar compounds modified by a PEG without reporting biological results.[16] Here we chose the latter strategy to improve the water solubility of the analogs of 'Pam$_3$CAG' and 'Pam$_2$CAG' by a PEG$_{2000}$. These new compounds were evaluated on mouse dendritic cell maturation, B cell proliferation and proved to involve the TLR2 pathway.

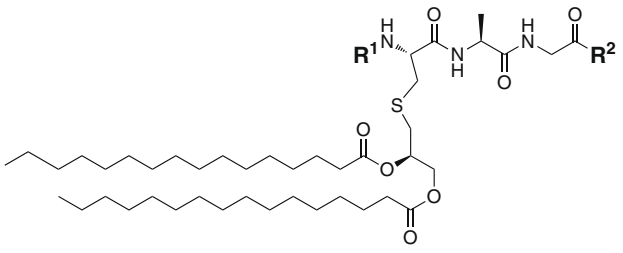
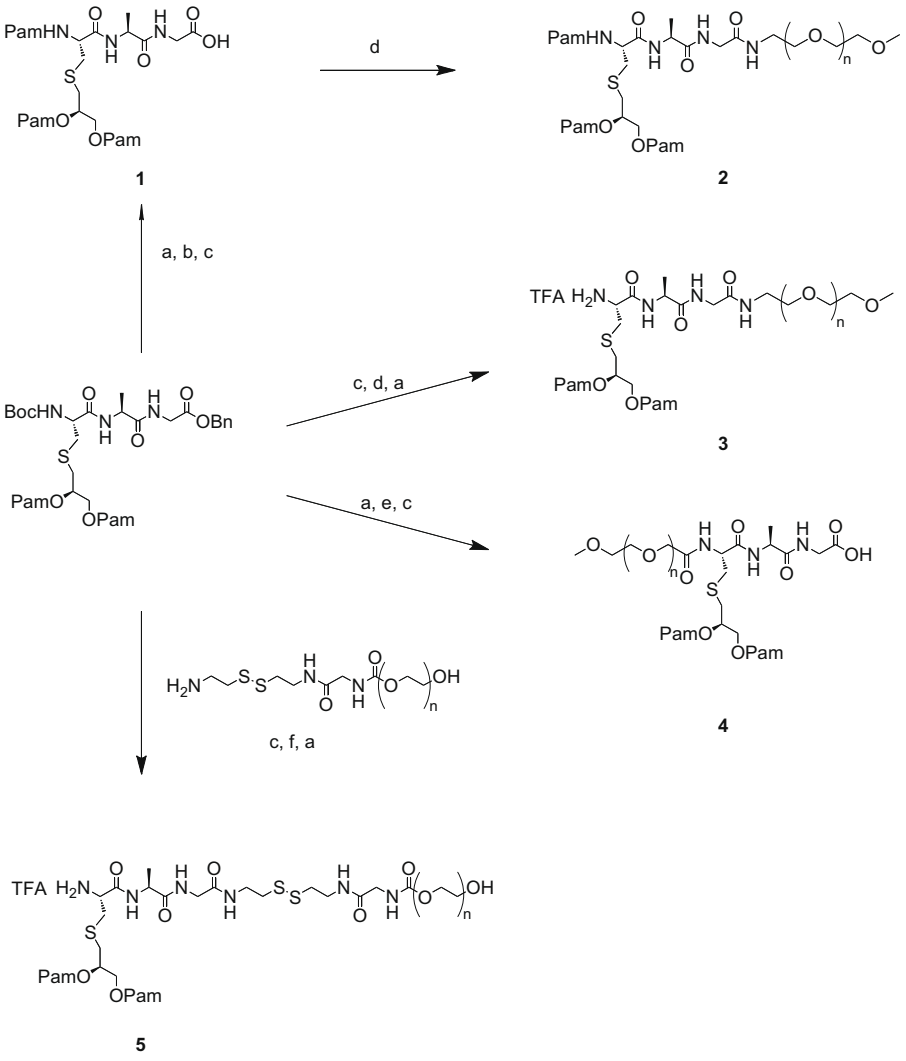


Figure 1. General structure for lipopeptides **1**–**5**. Compound **1**: Pam$_3$CAG-OH R^1 = Pam, R^2 = OH. Compound **2**: R^1 = Pam, R^2 = NH–(CH$_2$–CH$_2$–O)$_n$–CH$_2$–CH$_2$–OCH$_3$ ($n \sim 44$). Compound **3**: R^1 = H (TFA salt), R^2 = NH–(CH$_2$–CH$_2$–O)$_n$–CH$_2$–CH$_2$–OCH$_3$ ($n \sim 44$). Compound **4**: R^1 = CH$_3$O–(CH$_2$–CH$_2$–O)$_n$–CH$_2$–CO ($n \sim 44$), R^2 = OH. Compound **5**: R^1 = H (TFA salt), R^2 = NH–(CH$_2$)$_2$–S–S–(CH$_2$)$_2$–NH–CO–CH$_2$–NH–COO–(CH$_2$–CH$_2$–O)$_n$–CH$_2$–CH$_2$–OH ($n \sim 44$).

To introduce a PEG linker on *N*-α-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycine (**1**) and its analogs (Fig. 1), the accurate study of the molecule revealed that some parts could be modified more easily than others. To find which ones, we reconsidered the synthetic routes and the structure/activity relationships previously established.[17] Among the prominent modifications, an amide bond between the C-terminal function of **1** and the amine of a mono reactive PEG$_{2000}$ of the type H$_2$N–(CH$_2$–CH$_2$–O)$_n$–CH$_2$–CH$_2$–OCH$_3$ (where $n \sim 44$) was created to get compound **2** (Scheme 1). On the synthetic route to **1** (described as TLR2/TLR1 dimer ligand) is the diacylated *N*-α-*H*-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycine (improperly called 'Pam$_2$CAG', described as TLR2/TLR6 dimer ligand) where the N-terminal position is still free. In a previous



Scheme 1. Synthesis of PEG$_{2000}$ derivatives. Reagents and conditions: (a) TFA, CH$_2$Cl$_2$, 12 h, rt; (b) PamOH, DCC, DMAP, CH$_2$Cl$_2$, 6 h, rt; (c) H$_2$, Pd/C, CH$_2$Cl$_2$/MeOH, 12 h; (d) H$_2$NPEG$_{2000}$OMe, BOP, DIPEA, CH$_2$Cl$_2$, 12 h, rt; (e) MeOPEG$_{2000}$COOH, BOP, DIPEA, CH$_2$Cl$_2$, 12 h, rt; (f) BOP, DIPEA, CH$_2$Cl$_2$, 12 h, rt.

work, we demonstrated that this position is quite tolerant in contrast to the rest of the molecule, especially the cystein side chain, that needs to be at least double acylated in order to keep affinity for the lipid bilayer and immunoadjuvant activity.[18–20] Therefore, both free amine and corresponding amides, in which the amine was acylated by a non-fatty acid, have been described to remain active. As a consequence, we designed both compound **3** where an amide is created between the C-terminal function of 'Pam$_2$CAG' and the amine of the same mono reactive amino-PEG$_{2000}$ (*N*-α-*H*-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycinyl-NH-PEG$_{2000}$-OMe) and compound **4** where the N-terminal amine is acylated by a mono reactive PEG$_{2000}$ of the type CH_3O–$(CH_2$–CH_2–$O)_n$–CH_2–COOH (where $n \sim 44$), that is, *N*-α-PEG$_{2000}$-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycine. Finally, on the basis of compound **3** (with only two fatty chains), another analog, in which the PEG$_{2000}$ chain was linked to a disulfure bridge labile in proper red/ox conditions was designed. The rational for this modification lies on the prodrug concept[21] asserting that the hydrophilicity of compound **5** (*N*-α-*H*-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycinyl-NH–$(CH_2)_2$–S–S–$(CH_2)_2$–NH–CO–CH_2–NH–COO–$(CH_2$–CH_2–$O)_n$–CH_2–CH_2–OH, $n \sim 44$), was necessary for its bioavailability and could be removed at the expected site by reduction.

Thanks to a convergent strategy, four analogs of the above-mentioned 'Pam$_3$CAG' were thus synthesized (Scheme 1). For every compound, the first steps were identical until a common 'Pam$_2$-Cys-Ala-Gly-OH' scaffold was obtained (see Supplementary data). Briefly, the two carboxylic acids of (Boc-L-Cys-OH)$_2$ were esterified by benzyl alcohol under phase transfer conditions (water saturated by $NaHCO_3$, pH 8.5, CH_2Cl_2 as solvents and tricaprylylmethylammonium chloride as phase transfer agent). The SN_2 reaction was performed in 24 h at room temperature within 86% yield. Then, after a complete reduction by an excess of zinc for 2 h in acidic conditions, the thiol reacted on (*R*)-(+)-glycidol for 36 h at 40 °C within 31% yield to get a one pot thioether bond. At this step, the separation of unreacted (*R*)-(+)-glycidol from the expected compound was tricky. After purification, the two alcohols were acylated by palmitic acid after activation by DCC in the presence of DMAP within a 93% yield. Finally, the benzyl protection was removed under catalytic hydrogenation conditions (high pressure H_2 was used over Pd/C in a CH_2Cl_2/MeOH mixture) to get a first scaffold *N*-α-Boc-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteine within 88% yield and a 23% overall yield. The expected scaffold *N*-α-*H*-*S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-L-cysteinyl-L-alanyl-glycine was obtained by the convergent formation of an amide between the C-terminal and an alanyl-glycyl-derivative properly chosen. Syntheses of compounds **4** and **5** were then performed by peptidic couplings with the corresponding PEG$_{2000}$ moiety starting from this common scaffold. Reasonable yields were encountered, that is, **2**: 25% starting from 'Pam$_3$CAG', **3**: 31% from 'BocPam$_2$CAG', **4**: 35% from 'Pam$_2$CAG-OBn' and **5**: 27% from 'BocPam$_2$CAG'. All these compounds display an interesting solubility profile as they are soluble in dichloromethane and chloroform as apolar solvents, in methanol but above all, the four compounds are soluble in water (1 mg/mL).

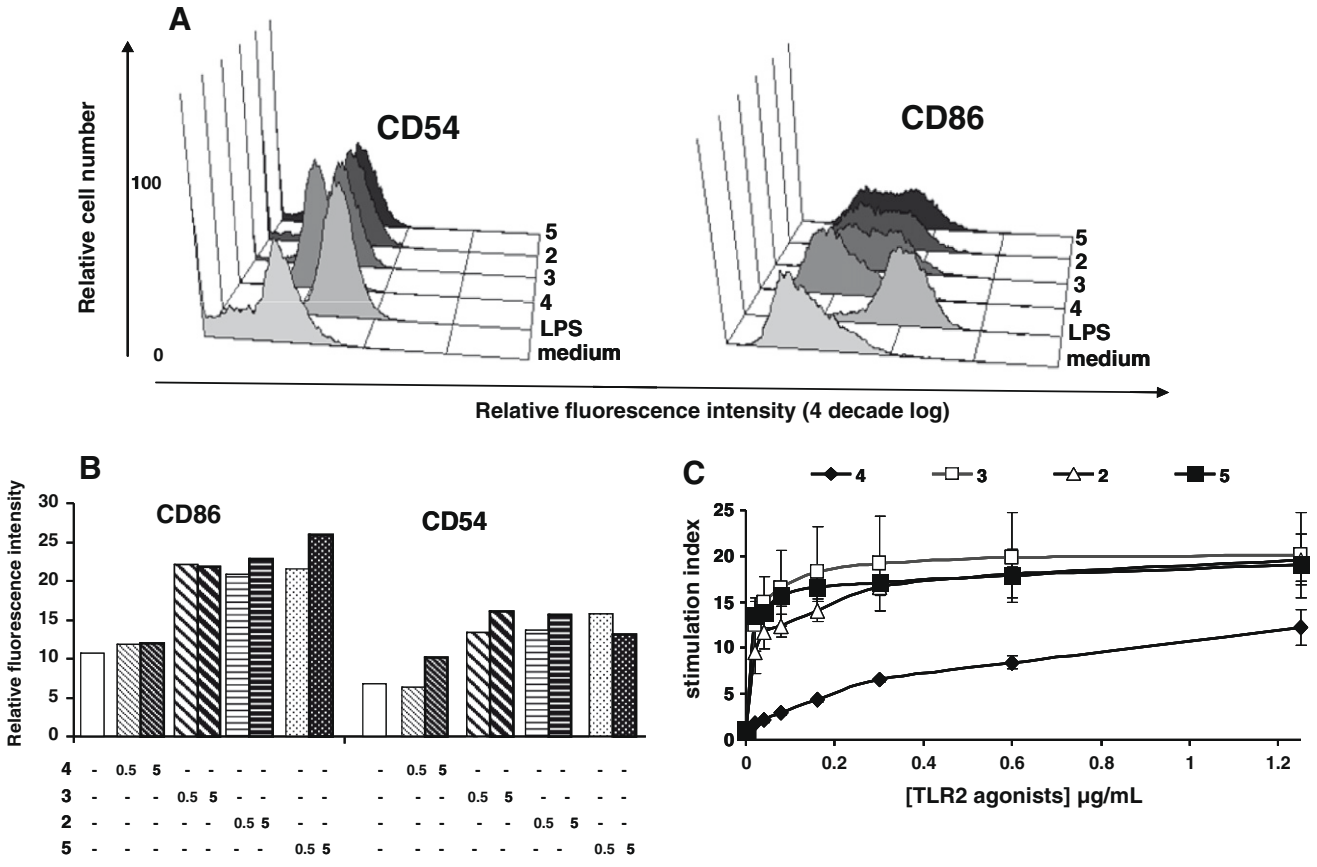


Figure 2. Water soluble analogs induce mouse dendritic cell maturation and mouse spleen B cell proliferation. (A, B) Maturation of D1 mouse dendritic cell line. Cells (2.10^5) were cultured for 48 h before addition of **2**, **3**, **4** or **5** analogs at 5 µg/ml (A, B) or 0.5 µg/mL (B). As positive control LPS (5 µg/mL) was used. After 24 h, expression of CD54 and CD86 activation markers was measured by flow cytometry. (A) One representative histogram and (B) MFI (mean fluorescence intensities) measured for each maturation marker (on experiment representative of 3). (C) Proliferation of mouse spleen B cells. Purified spleen B cells were cultured with the different analogs at the indicated concentration for 72 h. Proliferation was then evaluated by measuring the [3*H*]-thymidine uptake. Data are expressed as mean SI ± SD of three independent experiments (each experiment was performed in triplicate). SI = stimulation index which is the ratio of the average counts per minute in stimulated cells to the average counts per minute for unstimulated cells.

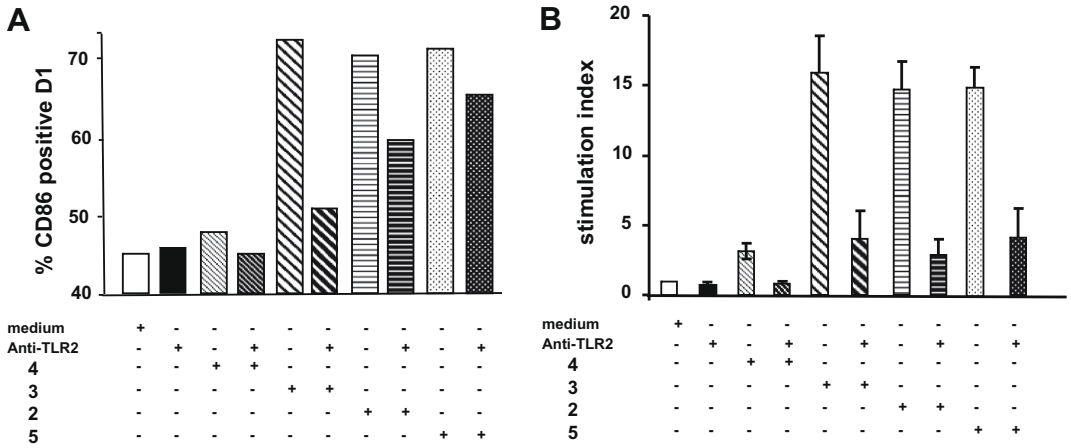


Figure 3. Water soluble analog-induced activation is decreased by antagonist anti-TLR2 antibody DC maturation (A) and B cell proliferation (B) were measured as in Figure 2 except that antagonist anti-TLR2 antibodies (clone HM1054) were added at 1 μg/mL 25 min before addition of the various water soluble analogs used at 0.5 μg/mL (A) or 0.16 μg/mL (B).

To evaluate if the water soluble analogs retain their biological activity, we checked their ability to induce mouse dendritic cell maturation[22] and mouse spleen B cell proliferation,[23] two well described biological effects of TLR2 agonists.

We investigated the capacity of the analogs to induce DC maturation by using the mouse DC line D1[24] which matures upon TLR signaling. As shown in Figure 2A and B, the four analogs induced an increase in the expression of the activation markers CD54 and CD86 at concentration ranging from 0.5 to 5 μg/mL. Whereas compounds **2**, **3** and **5** induced a maturation level similar to that induced by LPS, compound **4** is less efficient. For note, experiments using the water soluble analogs were performed in the presence of the LPS activity inhibitor polymyxin B to rule out the possibility of contaminating LPS D1 maturation.

We next investigated the capacity of the four analogs (2–5) to induce B cell proliferation. For this, B cells were isolated from mouse spleen and cultured in the presence of the analogs. Compounds **2**–**5** induced B cell proliferation (Fig. 2C) from the low concentration of 0.02 μg/mL. As for DC maturation, compound **4** was less efficient in inducing B cell proliferation.

Finally, to ensure that water soluble analogs induce DC maturation and B cell proliferation via TLR2 pathway, we repeated the experiments in the presence of antagonist anti-TLR2 antibody that inhibits the binding of TLR2 agonist to TLR2. As shown in Figure 3, the four analog activity was dramatically reduced in the presence of anti-TLR2 antagonist confirming that the biological activities of the water soluble analogs involve the TLR2 pathway.

In conclusion, we synthesized some new water soluble lipid immunoadjuvants. The biological results obtained with our innovative molecules on mouse dendritic cells as well as on mouse B cell showed that these compounds are still able to induce a strong immune response. We therefore demonstrated that they involve the TLR2 pathway. To the best of our knowledge, though 'Pam_3C' water-soluble analogs were already reported, it is the first time that 'Pam_2CAG' water-soluble analogs are described together with a biological characterization of their immunoadjuvant profile. This class of molecules will be incorporated in vaccine cocktails for cancer immunotherapy or infectious disease prevention, giving the opportunity to afford safer and more efficient vaccines, a point of crucial importance in the present health context.

Acknowledgements

We thank Patrick Wehrung and Pascale Buisine for ESI-MS. We gratefully acknowledge financial support from Université de Strasbourg, Centre National de la Recherche Scientifique (C.N.R.S.). J.B. and E.B. were granted by Region Alsace, ARC and Ministère de la Recherche, respectively.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.146.

References and notes

1. O'Hagan, D. T.; De Gregorio, E. *Drug Discovery Today* **2009**, *14*, 541.
2. Hecht, M.-L.; Stallforth, P.; Varon Silva, D.; Adibekian, A.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2009**, *13*, 354.
3. Bundle, D. R. *Nat. Chem. Biol.* **2007**, *3*, 605.
4. Bessler, W. G.; Suhr, B.; Büchring, H. G.; Müller, C. P.; Wiesmüller, K. H.; Becker, G.; Jung, G. *Immunobiology* **1985**, *170*, 239.
5. Jackson, D. C.; Lau, Y. F.; Le, T.; Suhrbier, A.; Deliyannis, G.; Cheers, C.; Smith, C.; Zeng, W.; Brown, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15440.
6. Aliprantis, A. O.; Yang, R. B.; Mark, M. R.; Suggett, S.; Devaux, B.; Radolf, J. D.; Klimpel, G. R.; Godowski, P.; Zychlinsky, A. *Science* **1999**, *285*, 736.
7. Takeda, K.; Kaisho, T.; Akira, S. *Annu. Rev. Immunol.* **2003**, *21*, 335.
8. Fernandes, I.; Frisch, B.; Muller, S.; Schuber, F. *Mol. Immunol.* **1997**, *34*, 569.
9. Roth, A.; Espuelas, S.; Thumann, C.; Frisch, B.; Schuber, F. *Bioconjugate Chem.* **2004**, *15*, 541.
10. Reichel, F.; Roelofsen, A. M.; Geurts, H. P. M.; van der Gaast, S. J.; Feiters, M. C.; Boons, G. J. *J. Org. Chem.* **2000**, *65*, 3357.
11. Voss, S.; Ulmer, A. J.; Jung, G.; Wiesmüller, K. H.; Brock, R. *Eur. J. Immunol.* **2007**, *37*, 3489.
12. Wermuth, C. G. In *The Practice of Medicinal Chemistry*; Wermuth, C. G., Ed., 3rd ed.; Academic Press: London, 2008; pp 767–785.
13. Commercially available at EMC Microcollections GmbH, Tübingen, Germany.
14. Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451.
15. Kleine, B.; Rapp, W.; Wiesmüller, K. H.; Edinger, M.; Beck, W.; Metzger, J.; Ataulakhanov, R.; Jung, G.; Bessler, W. G. *Immunobiology* **1994**, *190*, 53.
16. Heuking, S.; Iannitelli, A.; Di Stefano, A.; Borchard, G. *Int. J. Pharm.* **2009**, *381*, 97.
17. Spohn, R.; Buwitt-Beckmann, U.; Brock, R.; Jung, G.; Ulmer, A. J.; Wiesmüller, K. H. *Vaccine* **2004**, *22*, 2494.
18. Mühlradt, P. F.; Kiess, M.; Meyer, H.; Sussmuth, R.; Jung, G. *J. Exp. Med.* **1997**, *185*, 1951.
19. Mühlradt, P. F.; Kiess, M.; Meyer, H.; Sussmuth, R.; Jung, G. *Infect. Immun.* **1998**, *66*, 4804.
20. Metzger, J. W.; Beck-Sickinger, A. G.; Loleit, M.; Eckert, M.; Bessler, W. G.; Jung, G. *J. Pept. Sci.* **1995**, *1*, 184.
21. Stella, V. J.; Nti-Addae, K. W. *Adv. Drug Delivery Rev.* **2007**, *59*, 677.
22. Thoma-Uszynski, S.; Kiertscher, S. M.; Ochoa, M. T.; Bouis, D. A.; Norgard, M. V.; Miyake, K.; Godowski, P. J.; Roth, M. D.; Modlin, R. L. *J. Immunol.* **2000**, *165*, 3804.
23. Fillatreau, S.; Manz, R. A. *Eur. J. Immunol.* **2006**, *36*, 798.
24. Winzler, C.; Rovere, P.; Rescigno, M.; Granucci, F.; Penna, G.; Adorini, L.; Zimmermann, V. S.; Davoust, J.; Ricciardi-Castagnoli, P. *J. Exp. Med.* **1997**, *185*, 317.