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PII: S0040-4039(16)30317-3
DOI: http://dx.doi.org/10.1016/j.tetlet.2016.03.091
Reference: TETL 47479

To appear in: *Tetrahedron Letters*

Received Date: 9 March 2016
Revised Date: 24 March 2016
Accepted Date: 28 March 2016

Please cite this article as: Bazin, H.G., Bess, L.S., Livesay, M.T., Mwakwari, S.C., Johnson, D.A., Phospholipidation of TLR7/8-active imidazoquinolines using a tandem phosphoramidite method, *Tetrahedron Letters* (2016), doi: http://dx.doi.org/10.1016/j.tetlet.2016.03.091

Phospholipidation of TLR7/8-active imidazoquinolines using a tandem phosphoramidite method

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Keywords: TLR7/8, phospholipidation, imidazoquinoline, phosphoramidite

Abstract

A high-yielding and scalable phosphoramidite procedure was developed for the phospholipidation of TLR7/8-active imidazoquinolines. This method involves the reaction of a 1,2-diacyl- or dialkyl-*sn*-glycerol or 3-chlolesterylalkanol with 2-cyanoethyl *N*,*N*,*N*',*N*'-tetraisopropylphosphordiamidite in the presence of *1H*-tetrazole followed by treatment of the resulting *N*,*N*'-diisopropylphosphoramidite lipid in situ with 1-imidazoquinolinylalkanols. The resulting phosphite can be purified or directly oxidized with *t*-butyl hydroperoxide. The cyanoethyl protecting group is then removed with triethylamine and the phospholipidated imidazoquinoline products isolated in good yield and purity by simple filtration.

Toll-like receptors (TLR) are a family of more than 10 structurally related receptors on innate immune cells that detect pathogen-specific components common to large classes of microbial invaders. Activation of these receptors leads to the expression of inflammatory cytokines/chemokines and type I interferons (IFNα/β) important for effective innate and adaptive immune responses to infectious disease and cancer.

In the case of TLR7 and TLR8 activation, a few different classes of small-molecule mimetics of the natural uridine- and/or guanosine-rich viral ssRNA ligands have been identified,[1-3] including 1*H*-imidazo[4,5-c]quinolines[4] such as imiquimod (**1**, R-837; Fig. 1), which is approved

for topical treatment of certain skin diseases and known to primarily activate TLR7, and the structurally related imidazoquinoline resiquimod (**2**, R-848; Fig. 1), which potently activates both TLR7 and TLR8.[5] Since TLR7 and TLR8 are broadly expressed in DCs and other antigen presenting cells, TLR7/8 agonists and their derivatives may be especially useful as vaccine adjuvants. However, oral and topical preparations of imiquimod and resiquimod and other small-molecule TLR7/8 agonists can exhibit serious side effects, and clinical trials with certain TLR7/8 agonists have been suspended over safety concerns.[6,7] In addition, since TLR7 and TLR8 are located in endosomal/lysosomal compartments,[8] cellular uptake is prerequisite for cellular activation by TLR7/8 ligands. Thus, there is considerable interest in strategies that will increase the penetration of the TLR7/8 ligand into DCs and other immune cells as well as ameliorate toxic effects. Lipid conjugation of nucleoside drugs including TLR7/8 agonists[9] is one strategy known to facilitate endocytosis, enhance oral bioavailability, and decrease toxic side effects. Such nucleolipids can also be incorporated into liposomes and other biodegradable nanoparticles to help protect the drug from degradation and further reduce toxicity through a depot effect.[10]

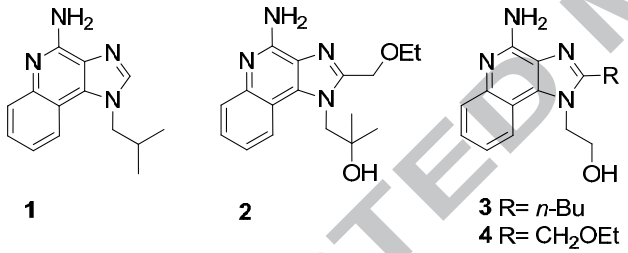


Figure 1. TLR7/8-active imidazoquinolines

Imidazoquinolines **3**[4] and **4**[4] were selected for initial phospholipidation studies due to their known TLR7/8 activity and simplified structures relative to resiquimod (**2**). Since the specific nature of the lipophilic moiety in the phospholipid conjugates is expected to influence both formulability and intracellular delivery, we investigated the conjugation of different lipid moieties to imidazoquinoline alcohols **3** and **4**, including cholesterol which is known to increase drug potency by binding specific membrane structures. We were particularly interested in developing an efficient synthesis of nucleolipids **5-8** (Fig. 2) that obviated the need to protect the primary amino group of the imidazoquinoline unit or isolate unstable phosphorous intermediates.

Here we report a high-yielding and scalable procedure for the phospholipidation of TLR7/8-active imidazoquinolines **3** and **4** using 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite (**11**) and various lipid alcohols in the key phosphitylation step.

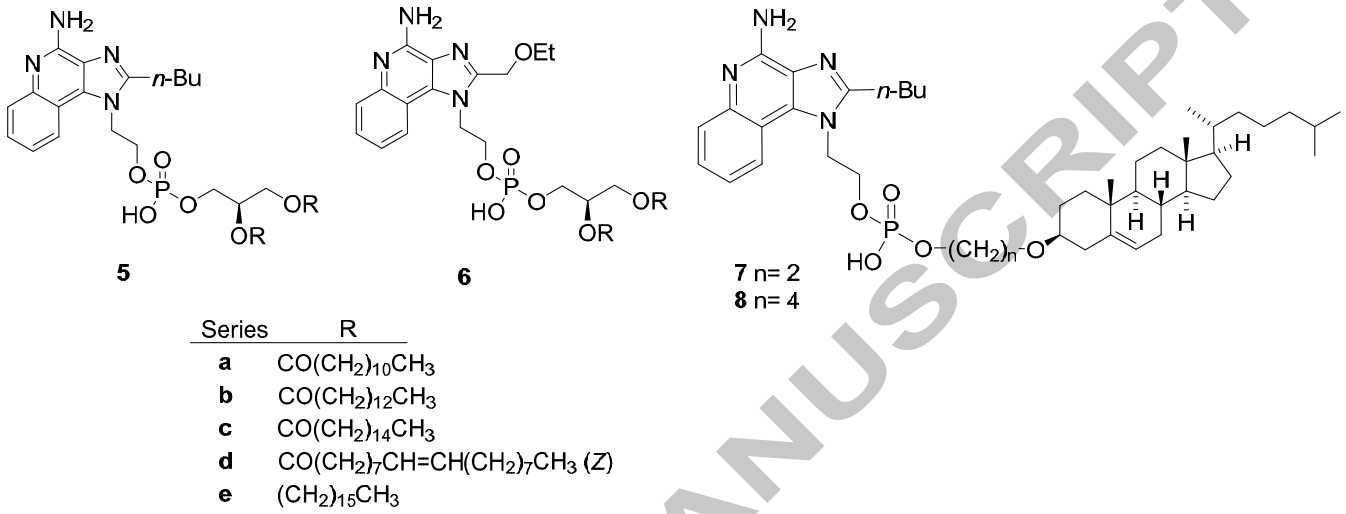
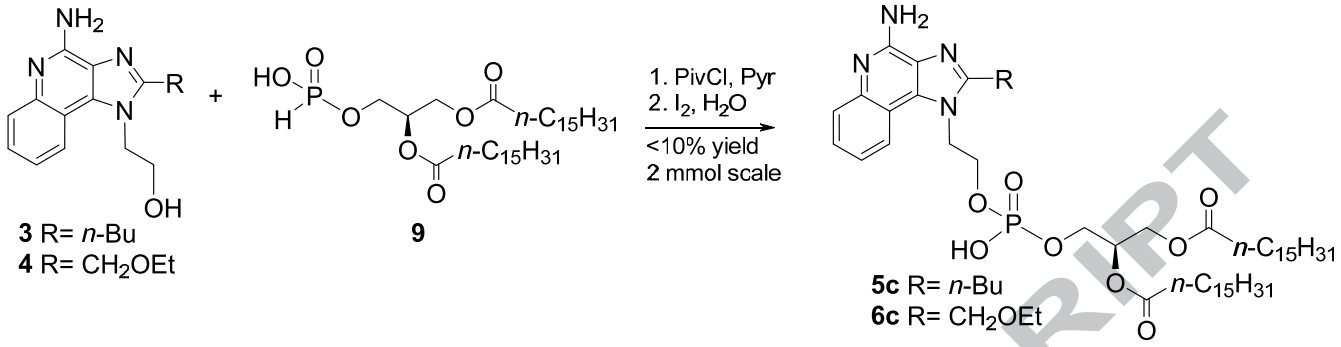


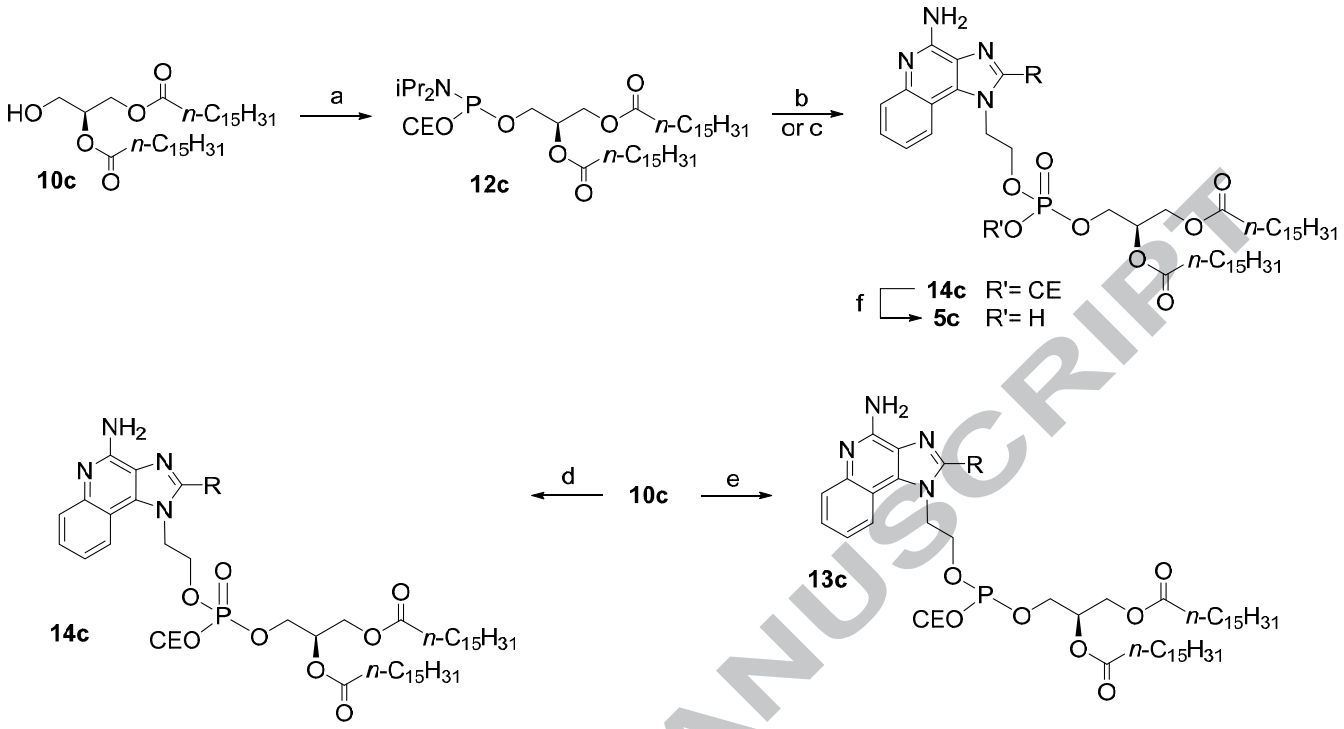
Figure 2. Phospholipid conjugates of imidazoquinolines **3** and **4**

Several procedures for the synthesis of phosphodiesters have been developed over the years using either phosphorous (III)[11,12] or phosphorous (V)[13-15] intermediates. Phosphorous (V) methods typically require long reaction times and lead to lower yields, whereas phosphorous (III) methods such as the hydrogen phosphonate (H-phosphonate)[11] and phosphoramidite[12] protocols are widely used for the synthesis of phosphodiesters and phosphotriesters. Accordingly, we first investigated the synthesis of nucleolipids **5c** and **6c** using the H-phosphonate method (Scheme 1). However, coupling of 1,2-palmitoyl-*sn*-glycero-3-H-phosphonate **9**[16] to imidazoquinolines **3** and **4** in the presence of pivaloyl chloride in pyridine under standard conditions (pre-concentration of starting materials from pyridine, anhydrous solvents and inert atmosphere), followed by oxidation of the resulting H-phosphonate diester with iodine, provided the desired phosphatidylated imidazoquinolines **5c** and **6c** in very low yield on preparative (2 mmol) scale, independent of the method used to prepare H-phosphonate **9** (PCl_3/imidazole,[16] salicylchlorophosphite[17] or phosphoric acid[18] methods) or the stoichiometry of the reagents. Because of the very low yields obtained with this method, we next investigated the synthesis of imidazoquinolines **5c** and **6c** using the phosphoramidite method (Scheme 2).



Scheme 1: Synthesis of phosphatidylated imidazoquinolines **5c** and **6c** via the H-phosphonate method

Reaction of 1,2-dipalmitoyl-*sn*-glycerol **10c** with a slight excess of commercially available 2-cyanoethyl *N*,*N*,*N'*,*N'*-tetraisopropylphosphordiamidite **11** and *1H*-tetrazole (Scheme 2a) led to phosphoramidite **12c** in 75% yield after chromatography on 1-g scale.[19] Subsequent coupling of **12c** (1.2 eq) with imidazoquinoline **3** in presence of dicyanoimidazole (DCI)[20] in acetonitrile followed by oxidation of phosphite intermediate **13c** (not isolated) with *m*-CPBA led to the desired phosphotriester **14c** (R=*n*-Bu) in 66% yield after purification by chromatography (Scheme 2b). Removal of the cyanoethyl (CE) group was effected with trimethylamine (TEA)[21] in acetonitrile. Most of the product precipitated out of solution in sufficiently pure form, obviating the need for chromatography, and **5c** was isolated by filtration in 53% overall yield. When this reaction was repeated using imidazoquinoline **4** and substituting DCI with imidazolium triflate[22] (Im-OTf) which is known to enhance O-selectivity in the phosphitylation step, and *m*-CPBA with *t*-butyl hydroperoxide,[21] **14c** (R=CH_2OEt) was obtained in 76% yield (Scheme 2c). This protocol eliminated the aqueous work-up required for *m*-CPBA removal and simplified the purification of **5c** from reagents and by-products. Nevertheless, this method was still hampered at larger scales (> 2.0 g) by low yield for the preparation of phosphoramidate **12c**, largely because of the instability of **12c** to chromatographic purification.

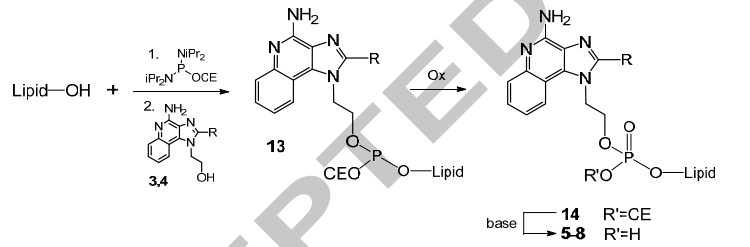


Scheme 2: Synthesis of phosphatidylated imidazoquinoline **5c** via phosphoramidite methods. *Reagents and conditions*: (a) **11**, *1H*-tetrazole (2 eq), CH_2Cl_2, rt, **12c** 75%; (b) (i) **3**, DCI (2.8 eq), CH_3CN; (ii) *m*-CPBA (1.5 eq), -10 ^oC, **14c** (R=*n*-Bu) 66%; (c)(i) **4**, Im-OTf (2 eq), CH_2Cl_2; (ii) *t*-BuO_2H, **14c** (R=CH_2OEt) 76%; (d) (i) **11** (2.1 eq), *1H*-tetrazole (2.1 eq), CH_2Cl_2, rt; (ii) **3**, Im-OTf (1.5 eq), 0 ^oC to rt; (iii) *t*-BuO_2H (1.5 eq), rt, **14c** (R=*n*-Bu) 62%; (e) (i) **11** (2.1 eq), *1H*-tetrazole (2.1 eq), CH_2Cl_2, rt; (ii) **3**, Im-OTf (1.5 eq), 0 ^oC to rt, **13c** (R=*n*-Bu) 93%; (f) TEA, CH_3CN, rt, **5c** (R=*n*-Bu) 80%.

This led us to investigate a one-pot procedure for the preparation of phosphotriester **14c** which would obviate the need to isolate unstable phosphoramidite intermediates such as **12c**. Initial experiments done by adding a stoichiometric amount of phosphoramidite **11** to a solution of 1,2-dipalmitoyl-*sn*-glycerol **10c** (1.0 eq) and *1H*-tetrazole (2.0 eq) followed by addition of imidazoquinoline **3** and Im-OTf, and subsequent oxidation of the intermediate phosphite **13c** in situ with *t*-butyl peroxide led to **14c** in 32% yield. Using DCI or Im-OTf in place of *1H*-tetrazole in the first coupling step led to lower yields of **14c** (27% and 13%, respectively). The two major by-products formed in the first coupling step resulted from the substitution of the diisopropylamine group of **12c** by a hydroxyl or another 1,2-dipalmitoyl-*sn*-glycerol moiety. Further optimization of this reaction was conducted by investigating the order of addition[23] and

reagents stoichiometry in the first coupling step. The highest yield (62%, scheme 2d)[24] of **14c** was obtained by (i) adding *1H*-tetrazole (2.1 eq) in several portions to a methylene chloride solution of 1,2-dipalmitoyl-*sn*-glycerol **10c** (2.0 eq) and phosphordiamidite reagent **11** (2.1 eq) and stirring 1 hour at room temperature, (ii) cooling the reaction mixture to 0 ^{o}C, adding imidazoquinoline **3** (1.0 eq) and Im-OTf (1.5 eq) and stirring at room temperature for 1 hour, followed by oxidation with *t*-butyl peroxide. Following this one-pot procedure, a hard-to-remove non-polar impurity (not isolated but believed to be a lipid by-product) was formed during the oxidation of **13c** to **14c**. Purification of the intermediate phosphite **13c** by flash chromatography (93% yield, scheme 2e) prior to oxidation eliminated the formation of this impurity. This optimized procedure was applied to the synthesis of phospholipids **5**-**8** from imidazoquinolines **3** and **4** and several different lipids in good to high yield on gram-scale (Table 1). The phosphotriesters **14** were subsequently deprotected using TEA in acetonitrile allowing the final phospholipidated imidazoquinolines **5**-**8** to be isolated in good yield and purity by simple filtration.[25]

Table 1. Phospholipidation of imidazoquinoline via the phosphoramidite method



Lipid	R	Yield%			Overall
		13	**14**	**5-8**	Yield%
1,2 dilauroyl-*sn*-glycerol	*n*-Bu	n.i.	79	77 (**5a**)	61
1,2 myristoyl-*sn*-glycerol	*n*-Bu	62	n.i.	89 (**5b**)	55
1,2 dipalmitoyl-*sn*-glycerol	*n*-Bu	93	n.i.	71 (**5c**)	66
	n-Bu	n.i.	62	72 (**5c**)	45
	CH_2OEt	p.p.	90	77 (**6c**)	69
	CH_2OEt	99	n.i.	73 (**6c**)	72
1,2-dioleoyl-*sn*-glycerol	*n*-Bu	92	n.i.	75 (**5d**)	69
1,2 dipalmityl-*sn*-glycerol	*n*-Bu	97	n.i.	71 (**5e**)	69
3-(2-hydroxyethyl)cholesterol	*n*-Bu	99	n.i.	44 (**7**)	44
3-(4-hydroxybutyl)cholesterol	*n*-Bu	96	n.i.	56 (**8**)	54

n.i. not isolated; p.p partially purified

In conclusion, we have developed a high-yielding and scalable procedure for the phospholipidation of imidazoquinolines which obviates the need to isolate unstable

phosphoramidite intermediates. The use of the cyanoethyl protecting group in the synthesis of the phosphotriester allows for an easy final deprotection and simple isolation of the corresponding phosphodiester in high purity by simple filtration. This simple and high-yielding phospholipidation procedure is applicable to other hydroxyl-containing biologically active molecules. The biological activity of these phospholipidated imidazoquinolines will be reported elsewhere.

Acknowledgments

This work was supported in part by the National Institute of Allergy and Infectious Diseases (NIAID) under contract HHSN272200900036C (to Corixa Corporation d/b/a GlaxoSmithKline Biologicals SA). Any opinions, findings, conclusions or recommendations expressed in this article are those of the authors and do not necessarily reflect the views of the NIAID.

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24. *General procedure for the synthesis of phosphotriester **14** via the phosphoramidite method*: the lipid (2.0 eq) and 2-cyanoethyl *N*,*N*,*N*',*N*'-tetraisopropylphosphordiamidite (2.1 eq) were dissolved in anhydrous methylene chloride (0.4 M) at rt. *1H*-tetrazole (2.1 eq) was added in four portions over 20 minutes and the reaction mixture stirred at room temperature for 1 hour. The reaction mixture was cooled to 0 ^{o}C, imidazoquinoline (1.0 eq) and imidazolium triflate (1.5 eq) were added, and the reaction mixture allowed to warm up to room temperature. The reaction was usually done after 1 hour at rt. The resulting phosphite **13** can be purified at this stage (after reducing the volume by concentration under vacuum) or subsequently oxidized by addition of *t*-butyl hydroperoxide (1.5 eq) to the reaction mixture and stirring at rt for 30 min. After completion of the oxidation, the reaction mixture

was concentrated under vacuum and purified by chromatography on silica gel to give the desired phosphotriester **14**.

25. *General procedure for the deprotection of the cyanoethyl protecting group:* the phosphotriester **14** was dissolved in acetonitrile (0.06 M). Triethylamine (acetonitrile:TEA 1:0.35 v:v) was added and the reaction mixture stirred at rt for 6 to 18 hours. Once the deprotection was complete, the reaction mixture was filtered over a Büchner filter and the isolated solid rinsed with acetonitrile and dried under high vacuum. **5a**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.09 (bs, 1H), 7.30 (t, *J* = 7.6 Hz, 1H), 7.09 (bs, 1H), 6.81 (bs, 1H), 5.14 (m, 1H), 4.42-4.78 (m, 4H), 4.31 (dd, *J* = 3.2, 12.0 Hz, 1H), 4.11 (dd, *J* = 6.6, 12.2 Hz, 1H), 3.93 (t, 2H, *J* = 6.0 Hz, 1H), 2.93 (bs, 2H), 2.20 (dd, *J* = 7.8, 15.0 Hz, 4H), 1.84 (m, 2H), 1.42-1.49 (m, 6H), 1.15 (m, 32H), 0.96 (t, *J* = 7.2 Hz, 3H), 0.78 (t, *J* = 6.6 Hz, 6H); negative ES TOF-MS calc for $[M-H]^-$ 801.4931, found 801.4858; **5b**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.17 (bs, 1H), 7.39 (bs, 1H), 7.17 (bs, 1H), 6.91 (bs, 1H), 5.24 (m, 1H), 4.42-4.87 (m, 4H), 4.41 (dd, *J* = 3.6, 12.4 Hz, 1H), 4.19 (dd, *J* = 6.4, 12.0 Hz, 1H), 4.03 (t, *J* = 6.0 Hz, 2H), 2.99 (bs, 2H), 2.30 (m, 4H), 1.94 (bs, 2H), 1.51-1.59 (m, 6H), 1.25 (m, 40H), 1.05 (t, *J* = 7.2, 3H), 0.88 (m, 6H); negative ES TOF-MS calc for $[M-H]^-$ 857.5558, found 857.5565; **5c**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.23 (bs, 1H), 7.39 (t, *J* = 7.4 Hz, 1H), 7.22 (bs, 1H), 6.93 (bs, 1H), 5.25 (m, 1H), 4.42-4.81 (m, 4H), 4.42 (dd, *J* = 3.2, 12.0 Hz, 1H), 4.19 (dd, *J* = 6.4, 12.0 Hz, 1H), 4.04 (t, *J* = 6.0 Hz, 2H), 3.06 (bs, 2H), 2.32 (m, 4H), 1.96 (m, 2H), 1.53-1.60 (m, 6H), 1.26 (m, 48H), 1.07 (t, *J* = 7.2 Hz, 3H), 0.88 (m, 6H); positive ES TOF-MS calc for $[M+H]^+$ 915.6340, found 915.6309; **5d**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.18 (bs, 1H), 7.39 (bs, 1H), 7.18 (bs, 1H), 6.92 (bs, 1H), 5.29-5.34 (m, 4H), 5.21-5.27 (m, 1H), 4.43-4.90 (m, 4H), 4.41 (dd, *J* = 3.2, 12.0 Hz, 1H), 4.19 (dd, *J* = 6.4, 12.0 Hz, 1H), 4.03 (t, *J* = 6.0 Hz, 2H), 3.01 (bs, 2H), 2.30 (m, 4H), 1.94-2.01 (m, 10H), 1.52-1.61 (m, 6H), 1.27 (m, 40H), 1.05 (t, *J* = 7.2, 3H), 0.88 (m, 6H); negative ES TOF-MS calc for $[M-H]^-$ 965.6497, found 965.6498; **5e**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.23 (bs, 1H), 7.40 (t, *J* = 7.6 Hz, 1H), 7.18 (bs, 1H), 6.96 (bs, 1H), 4.51-4.82 (m, 4H), 3.95 (t, *J* = 6.0 Hz, 2H), 3.55-3.67 (m, 4H), 3.37-3.52 (m, 3H), 3.03 (bs, 2H), 1.94 (m, 2H), 1.52-1.61 (m, 6H), 1.26 (m, 52H), 1.06 (t, *J* = 7.2 Hz, 3H), 0.88 (t, 6H); negative ES TOF-MS calc for $[M-H]^-$ 885.6598, found 885.6536; **6c**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ8.05 (bs, 1H), 7.29 (t, *J* = 7.6 Hz, 1H), 7.09 (bs, 1H), 6.78 (bs, 1H), 5.11 (m, 1H), 4.40-4.90 (m, 4H), 4.28 (dd, *J* = 3.6, 11.6 Hz, 1H), 4.07 (dd, *J* = 6.4, 11.6 Hz, 1H), 3.90 (t, *J* = 6.0 Hz, 2H), 3.54 (dd, *J* = 7.2 Hz, 2H), 2.18 (m, 4H), 1.47 (m, 4H), 1.16 (m, 51H), 0.76 (m, 6H); positive ES TOF-MS calc for $[M+H]^+$ 917.6132, found 917.6162; **7**: ^{1}H NMR (400 MHz, $CDCl_3$) δ8.25 (d, *J* = 7.68 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.20 (t, *J* = 7.6 Hz , 1H), 6.95 (d, *J* = 7.6 Hz, 1H), 5.30 (m, 1H), 4.58-4.81 (m, 4H), 4.02 (m, 2H), 3.68 (t, *J* = 5.6 Hz, 2H), 3.26 (m, 1H), 3.04 (dd, *J* = 8.4, 12.8 Hz, 2H), 2.36 (bd, 1H), 2.18 (m, 1H), 0.87-2.03 (m, 45H), 0.68 (s, 3H); positive ES TOF-MS calc for $[M-H]^-$ 775.4933, found 775.4870; **8**: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ 8.07 (bs, 1H), 7.31 (bs , 1H), 7.09 (bs, 1H), 6.88 (bs, 1H), 5.32 (m, 1H), 4.43-4.92 (m, 4H), 3.92 (m, 2H), 3.48 (m, 2H), 3.11 (m, 2H), 2.94 (m, 1H), 2.32 (m, 1H), 2.16 (m, 1H), 0.86-2.02 (m, 49H), 0.68 (s, 3H); negative ES TOF-MS calc for $[M-H]^-$ 803.5240, found 803.5195.

Phospholipidation of TLR7/8-active imidazoquinolines using a tandem phosphoramidite method

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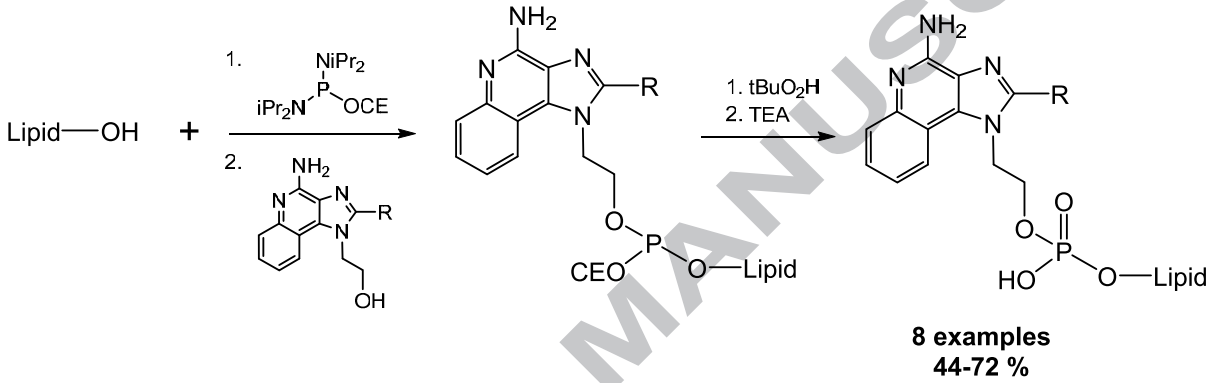

Highlights

- A high-yielding and scalable phospholipidation procedure is described
- This phospholipidation procedure involves a tandem phosphoramidite method
- This procedure is used for the phospholipidaton of TLR7/8 active imidazoquinolines