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Synthesis, evaluation and incorporation into liposomes of 4-functionalised-2,5-diphenyloxazole derivatives for application in scintillation proximity assays

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Abstract—The preparation of a series of 4-functionalised-2,5-diphenyloxazoles is described. The scintillating efficiency of each of these 'scintilipid' molecules has been evaluated in the presence of ionising radiation. Each 'scintilipid' has been assessed for the ability to assemble, with other lipids, into liposomes, under a variety of preparative conditions. Each liposomal preparation has been monitored for the ability to scintillate in the presence of ionising radiation. The optimal 'scintilipid', both in terms of effective liposomal formation and scintillation efficiency, has been determined.

The scintillation proximity assay (SPA), developed and marketed by Amersham Biosciences, is a real-time, quantitative and dynamic assay for the evaluation of receptor/ligand binding interactions.[1] In a typical SPA procedure, the receptor is labelled by immobilisation upon the surfaces of SPA beads, microspheres that encapsulate scintillant molecules, whilst the ligand is radiolabelled with a short pathlength emitter such as tritium. When the ligand is bound by the receptor, the radiolabel is brought into close proximity with the scintillant molecules within the SPA bead resulting in the emission of light. The emitted light may be detected readily and quantified in a scintillation counter. If no binding occurs, the majority of the radioactivity remains too remote from the encapsulated scintillant to elicit a significant signal. The SPA has also been adapted to the study of whole cells in microtitre plate format.[2] Here, scintillant molecules, such as 2,5-diphenyloxazole, are incorporated into the plastic comprising the base of the microtitre plate. Cells are then grown on the bottom surfaces of the plate and incubated with a radiolabelled molecule. If the cells in a particular well take-up the radiolabelled molecule the base of the well will emit light in an analogous fashion to the bead-based assay.[2]

As part of our on-going program investigating scintillation phenomena, we were intrigued by the possibility of creating cells, which functioned directly as 'living SPA beads'. We reasoned that liposomal delivery of appropriately derivatised scintillant molecules should enable their incorporation into the membranes of living cells. Herein, we report the synthesis of a series of 4-functionalised-2,5-diphenyloxazoles and the evaluation of each derivative in terms of its scintillation efficiency.[3] We also report the suitability of each derivative for use in forming liposomes under standard conditions[4] and, in each case, have determined the scintillating efficiency of the resultant liposomes.

Previous work[3,5] has demonstrated that 2,5-diphenyloxazole **1** may be converted into 2,5-diphenyl-4-hydroxymethyloxazole **3** and 4-bromomethyl-2,5-diphenyloxazole **4** relatively easily. In addition, ethyl-2,5-diphenyl-4-oxazolecarboxylate **2** may be synthesised readily.[3] Scheme 1 outlines the divergent synthetic strategy that has been adopted, using these readily available precursors, to access a wide number of different 4-functionalised-2,5-diphenyloxazoles ('scintilipids') as efficiently as possible.

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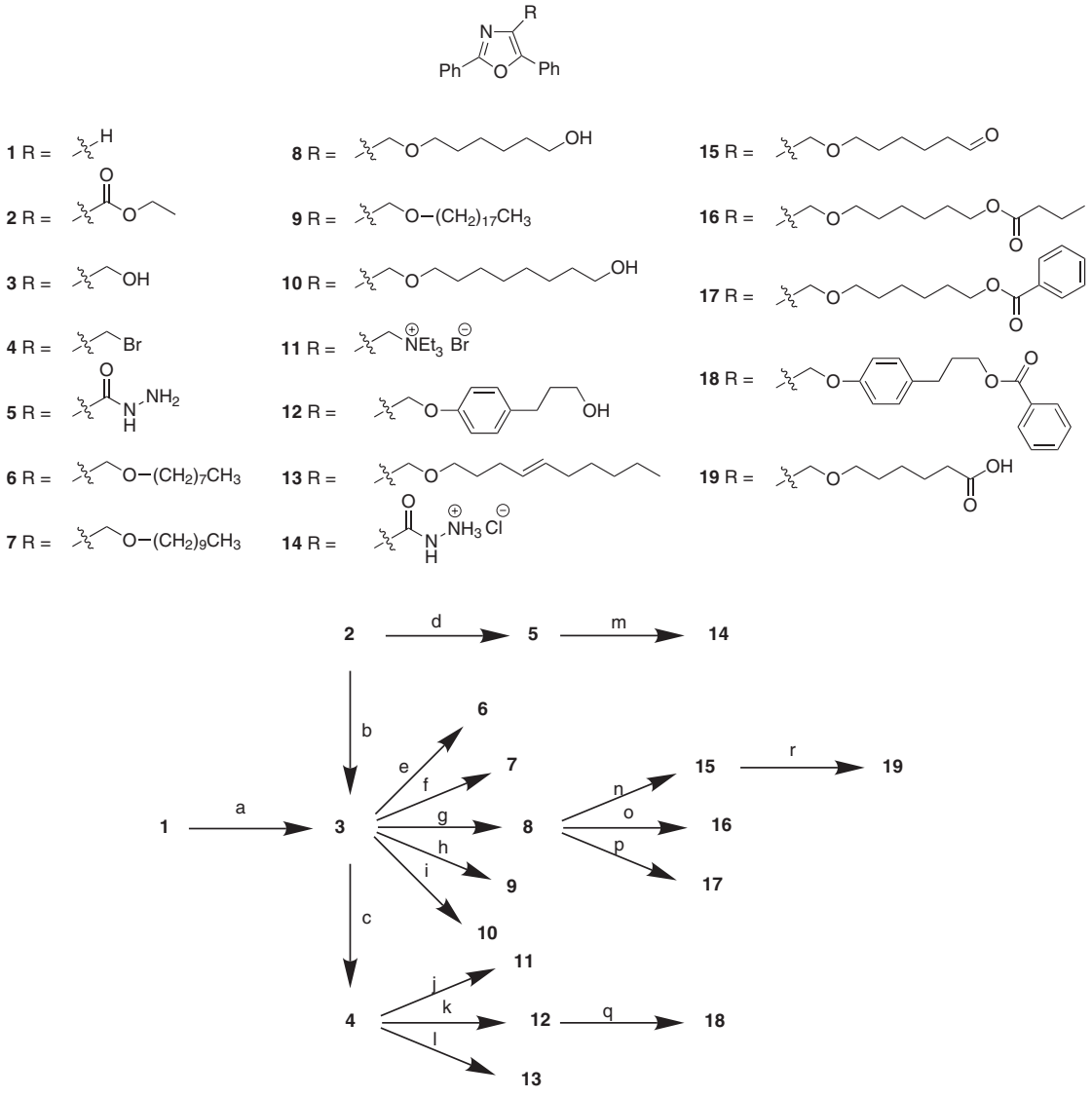

Scheme 1. Reagents and conditions: (a) (1) *sec*-BuLi, TMP, THF, −78 to 0 °C, DMF/10 h, rt; (2) $NaBH_4$, MeOH, 33%. (b) $LiBH_4$, $LiEt_3BH$, THF, 98%. (c) PBr_3, DCM, 98%. (d) $NH_2NH_2{\cdot}_xH_2O$, EtOH, 43%. (e) NaH, $C_8H_{17}Br$, DMF, 76%. (f) NaH, $C_{10}H_{21}Br$, DMF, 72%. (g) (1) NaH, $C_6H_{12}BrOSiBu^tMe_2$, DMF; (2) TBAF, THF, 40%. (h) NaH, $C_{18}H_{37}Br$, DMF, 76%. (i) (1) NaH, $C_8H_{17}BrOSiBu^tMe_2$, DMF; (2) TBAF, THF, 28%. (j) Et_3N, CH_3COCH_3, 66%. (k) $HOPh(CH_2)_3OH$, K_2CO_3, 18-crown-6, CH_3CN, 55%. (l) NaH, *trans*-$CH_3(CH_2)_4CH{=}CH(CH_2)_3OH$, DMF, 12%. (m) $HCl_{(g)}$, $CHCl_3$, 100%. (n) PCC/silica gel (1:1), DCM, 84%. (o) $CH_3(CH_2)_2COCl$, Et_3N, DCM, 45%. (p) PhCOCl, Et_3N, DCM, 52%. (q) PhCOCl, Et_3N, DCM, 65%. (r) Ag_2O, $NaOH_{(aq)}$, MeOH, 77%.

The fusion of a cell membrane with a liposome is facilitated when the liposome is constructed from cationic lipids.[6] Accordingly, the first target was the cationic tetraalkylammonium salt **11** that was synthesised, in one step, by treating a solution of bromide **4** in acetone with triethylamine. A second cationic scintillant-containing compound was synthesised by treating a solution of ester **2** in ethanol with excess hydrazine hydrate to generate hydrazide **5**. Subsequently, excess hydrogen chloride gas was bubbled through a solution of this hydrazide in chloroform to precipitate the hydrochloride salt **14**.

Unfortunately, hydrazide **5** and both scintillant-containing cationic compounds **11** and**14** and their corresponding liposomes ('scintiliposomes') all exhibited relatively low scintillation counting efficiencies in the presence of ionising radiation (Table 1). A possible explanation for the low scintillation efficiencies may be the close proximity of the oxazole ring to the electron density of the carbonyl group or a formal charge, which may cause quenching of scintillation through a charge transfer process. The prediction of scintillation efficiency in these systems is nontrivial and is highly dependent upon the type of chemical functionality attached at the 4-position of the oxazole. For example, previous work[3a] had shown that ester **2** scintillates with very high efficiency but aldehydic and carboxylic acid functionality at the 4-position of the 2,5-diphenyloxazole skeleton results in very poor scintillation.

In previous work[3a] an ether linkage had been used to attach substituents at the 4-position of 2,5-diphenyloxazole, whilst retaining very high scintillation counting

Table 1. Scintillation efficiencies of scintilipids **5**–**14**, **16**–**19** and scintillation counting data for scintiliposomes incorporating these lipids

Scintilipid	Scintillation efficiency[a,b]	Scintillation counts obtained for each liposomal composition/cpm		
		PC[a,c]	DOTAP[a,c]	DOTAP/DOPE (1:1)[a,c]
5	0.6	3	3	10
6	49	4	8	11
7	47	73	12	11
8	0.9	96	76	62
9	43	60	285	199
10	21	161	129	172
11	0.1	12	10	8
12	14	214	469	506
13	19	308	128	518
14	0.5	12	8	9
16	17	18	30	44
17	13	9	20	66
18	11	8	234	146
19	93	12	58	20

[a] Values are background corrected.

[b] To a solution of scintilipid compound in toluene (200 μL, 20 mM) was added [^{3}H]hexadecane (200 μL, 0.5 μCi/mL), followed by monitoring in a scintillation counter. Values correspond to the cpm as a percentage of the cpm detected with the addition of commercial scintillation fluid Microscint 20. All values have been adjusted to accommodate the fact that a system based solely on 2,5-diphenyloxazole **1** gives 70% scintillation counting efficiency when compared with commercial scintillation fluid.[3a]

[c] [^{14}C]taurine (0.1 μCi) was added to the scintiliposome preparation (500 μL, 10 mg/mL, 10:1, lipid/scintilipid) and the assay solution monitored in a scintillation counter.

efficiency in the presence of ionising radiation. The ether linkage was also deemed attractive due to its chemical robustness and ready accessibility via either scintillant alcohol **3** or scintillant bromide **4**. Accordingly, all compounds synthesised subsequently, that is compounds **6**–**10**, **12**, **13** and **15**–**19**, were constructed with an ether linkage between the 4-position of the 2,5-diphenyloxazole unit and the side chain.

Alcohol **12** was synthesised successfully by a potassium carbonate/18-crown-6 mediated Williamson-type ether synthesis between 3-(4-hydroxyphenyl)-propan-1-ol and bromide **4**. Alcohol **12** exhibited a modest but a sufficiently high scintillation efficiency (Table 1) for further evaluation. Accordingly, alcohol **12** was incorporated into standard liposomal preparations to provide the corresponding 'scintiliposomes'. These scintiliposomes, **12**/dioleoyltrimethylammonium propane (DOTAP) (469 cpm), **12**/phosphatidylcholine (PC) (214 cpm) and **12**/DOTAP/dioleoylphosphatidylethanolamine (DOPE) (506 cpm) exhibited a significant number of scintillation counts compared with all other scintiliposome preparations evaluated (Table 1). Unfortunately, microscopic examination of liposomal preparations incorporating alcohol **12** revealed crystalline regions of this scintilipid within all three scintiliposomes, which rendered them unusable in subsequent cell-based assays.

In an attempt to circumvent the problem of crystallisation within the scintiliposomes, alcohol **12** was modified further. Esterification with benzoyl chloride under standard conditions gave ester **18** in good yield. Ester **18** and scintiliposomes incorporating this scintilipid exhibited relatively high scintillation counting efficiencies but, unfortunately, microscopic examination of the scintiliposomes again revealed crystalline regions of the scintilipid.

In a further attempt to try and prevent the deposition of crystalline scintillant within the liposomes, the relatively rigid aromatic-containing side chains, present in scintilipids **12** and **18**, were replaced with side chains that were entirely aliphatic to give scintilipids **6**, **7** and **9**. Each scintilipid **6**, **7** and **9** was constructed in a Williamson-type ether synthesis reaction between alcohol **3** and the appropriate alkyl halide. The resultant scintilipids all exhibited high scintillation counting efficiencies (Table 1). Unfortunately, incorporation of scintilipids **6**, **7** and **9** into liposomes resulted, in each case, in a significant reduction in scintillation counting efficiency and the deposition of crystalline scintilipid within the liposomes.

To try and circumvent the problem of crystallisation it was next decided to evaluate a scintilipid with a degree of unsaturation in the side chain. A Williamson-type ether synthesis between *trans*-4-decenol (obtained in quantitative yield by reducing a sample of commercially available *trans*-4-decenal with sodium borohydride) and scintillant bromide **4** furnished scintillant alkene **13**. Whilst the yield for this reaction was disappointingly low (12%), sufficient material was produced for evaluation purposes. Alkene **13** exhibited only a moderate scintillation counting efficiency (19%) in the presence of ionising radiation. However, incorporation of the scintillant alkene **13** into liposome preparation provided scintiliposomes **13**/DOTAP (128 cpm), **13**/PC (308 cpm) and **13**/DOTAP/DOPE (518 cpm) that exhibited a significant number of scintillation counts compared with other liposome preparations evaluated (Table 1). In fact, **13**/PC and **13**/DOTAP/DOPE exhibited greater numbers of counts than any other scintiliposomes, of analogous composition, that were evaluated. Unfortunately, microscopic analysis revealed that each scintiliposome contained crystalline regions of scintilipid **13**.

In a further attempt, to prevent crystallisation of the scintilipids within the liposomes, a set of scintilipids that contained functionalised but fully saturated side chains were prepared. Alcohol **8** was synthesised by a Williamson-type ether synthesis between a silylated derivative of 6-bromo-1-hexanol and scintillant alcohol **3**. Initially, the hydroxyl functionality of 6-bromo-1-hexanol was protected by silylation with *tert*-butyldimethylsilyl chloride. The resultant silyl-ether was added to a solution of scintillant alkoxide in DMF, generated by treatment of **3** with sodium hydride, to give the coupled scintillant silyl-ether adduct, which was then desilylated by treatment with tetrabutylammonium fluoride (TBAF) to provide alcohol **8**. Surprisingly, alcohol **8** gave only a low scintillation counting efficiency (0.9%) but still furnished liposomal preparations, which gave significant scintillation counts, **8**/PC (96 cpm), **8**/DOTAP (76 cpm), **8**/DOTAP/DOPE (62 cpm). This encouraging result prompted the derivatisation of alcohol **8** into a number of other scintillant-containing compounds **15**, **16** and **17**.

Long, linear aliphatic carboxylic acids dissolved in a solvent above their critical micelle concentration frequently assemble into micelles.[7] Consequently it was anticipated that 2,5-diphenyloxazole tagged with an aliphatic carboxylic acid moiety would be incorporated readily into liposomes. Attempts to oxidise alcohol **8** directly into the corresponding carboxylic acid **19** using either Jones reagent (CrO_3/AcOH) or $KMnO_4$ generated a variety of oxidised products. Consequently, a less vigorous two-step oxidation strategy was employed to convert alcohol **8** into carboxylic acid **19** successfully via intermediate aldehyde **15**. Contrary to a previous finding that indicated that carboxylic acids quench scintillation significantly,[3a] carboxylic acid **19** exhibited the greatest scintillation efficiency (93%) of all the scintilipids evaluated! Unfortunately, incorporation of the carboxylic acid **19** into various liposomal preparations produced scintiliposomes that only exhibited comparatively low scintillation counts (Table 1).

Alcohol **8** was further derivatised by esterification with butyryl chloride and benzoyl chloride to provide the corresponding esters **16** (45%) and **17** (52%), respectively. Esters **16** and **17** have scintillation counting efficiencies (17% and 13%, respectively) that are approximately 20 and 15 times greater than alcohol **8** (0.9%). Unfortunately however, incorporation of either of these esters into liposomal preparations gave liposomes that exhibited scintillation counts below those obtained for liposomes incorporating alcohol **8**.

Since derivatives of alcohol **8** gave no significant improvements over using the precursor alcohol, an attempt was made to investigate an analogue of alcohol **8**, which differed only in the length of the alkyl chain between the hydroxyl group and the oxazole ring. Accordingly, a sodium hydride mediated Williamson-type ether synthesis reaction between scintillant alcohol **3** and silylated-8-bromo-1-octanol, followed by TBAF desilylation of the product, gave alcohol **10**, which has an aliphatic chain length two methylene units longer in length than alcohol **8**. Gratifyingly, alcohol **10** exhibited a scintillation counting efficiency (21%) that is 25 times greater than alcohol **8** (0.9%). More importantly however, alcohol **10** produced approximately twice the number of scintillation counts, compared with **8**, when incorporated into liposomal preparations. Scintiliposomes incorporating alcohol **10** have been used to introduce scintilipid **10** into the membrane of living HeLa cells. These cells have been used successfully in a cell-based SPA to detect and quantify the uptake of [^{14}C]methionine.[4]

In conclusion, we have derivatised the 4-position of 2,5-diphenyloxazole systematically to generate a series of scintilipids. It has been demonstrated that through an iterative process of synthesis, assay and evaluation it has been possible to develop a 'lipid-like' scintillant-containing molecule that scintillates efficiently in the presence of ionising radiation either in solution or within liposomal membranes. Currently we are investigating further cell-based SPA style assays employing scintilipid **10** and will report our findings in due course.

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