Tetrahedron Letters, Vol.27, No.3, pp 271-274, 1986 0040-4039/86 $3.00 + .00
Printed in Great Britain

USE OF PHOSPHOTRIESTER SYNTHETIC METHODS FOR PREPARATION OF PHOSPHATIDYLETHANOLAMINE-ANALYTE CONJUGATES

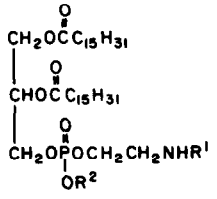
Say-Jong Law, Arthur Myles*
Department of Molecular Biology and Immunology
Collaborative Research, Inc.
Lexington, MA 02173

Abstract: Amphiphilic phosphatidylethanolamine conjugates of therapeutically or biologically active analytes have been prepared using a new phosphotriester synthetic approach and used for functionalization of liposomes.

Liposomes are small single or multilamellar synthetic vesicles which have many of the properties of cells or biological membranes. They have been investigated extensively as drug delivery systems, as immunogens and as model systems for the study of complement mediated membrane cytolysis[1-3]. The latter is of particular interest since liposomes containing specific surface bound antigens are susceptible to complement mediated damage in the presence of cognate antibody[4]. As such they provide a basis for an immunodiagnostic assay system for quantitation of analytes in biological fluids[5-9]. Further expansions of these concepts in our labs has resulted in development of a homogeneous colorimetric assay for penicillin and thyroxine. Enhanced signal generation was provided by construction of antigen tagged liposomes encapsulating an enzyme[10]. These enzyme membrane immuno assays for specific antigens, carried out in the presence of appropriate external substrate, are readily monitored visually or spectrophotometrically giving highly sensitive and reproducible results. These assays are rapid, homogenous and provide a nonisotopic assay method with sensitivity and reproducibility equivalent to radioimmunoassay[11].

One of the major problems associated with development of such an assay system for small molecule analytes was synthesis of phospholipid-analyte conjugates for preparation of suitably functionalized liposomes. Traditionally, such conjugates have been synthsized either by direct coupling of phosphatidylethanolamine (PE) or phosphatidic acid with the analyte[12,13] or by coupling procedures with bifunctional cross linking reagents[14]. Other potential preparative routes involve complex syntheses requiring partial or complete construction of the PE backbone[15-17]. None of the procedures are suitable for our purposes. The direct methods give multiple products which are difficult to separate and characterize. In addition, many analytes are not chemically compatible for direct reaction with PE but require use of a ligand to facilitate coupling. The total synthetic approaches are too complicated for the number and variety of analyte conjugates required.

We therefore developed a simple but flexible synthetic strategy involving use of PE phosphotriester intermediates with easily removable triester blocking groups. These are prepared from commercially available PE. Conjugation of analytes or ligands with these phosphotriesters occurs readily to give products of defined composition which are simpler to purify and characterize. The partially or fully protected conjugates are converted, via base catalyzed reactions, to the unprotected PE-analyte or PE-ligand-analyte phosphodiester conjugates as required for construction of analyte functionalized liposomes. We wish to report the synthesis of protected PE phosphotriester intermediates and their direct coupling to penicillin G and indirect coupling, via a succinamide ligand, to L-thyroxine[18]. The deprotected conjugates have been used for preparation of analyte functionalized liposomes which form the basis of highly reproducible and sensitive assays for penicillin and thyroxine[19, 11]. The synthetic procedures are also currently being used for preparation of other PEA analyte conjugates for expansion of this technology. Similar conjugates could also have potential in funtionalizing liposomes for use as immunogens or for enhancing targeting of liposome based drug delivery systems.

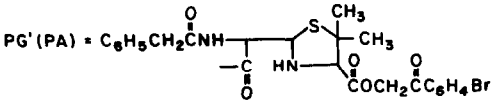


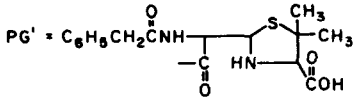
Tr = $-C(C_6H_5)_3$

BOC = $-COC(CH_3)_3$

NPE = $-CH_2CH_2C_6H_4NO_2$

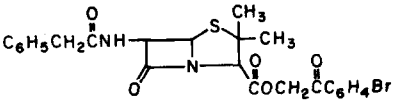
PA = $-CH_2CC_6H_4Br$



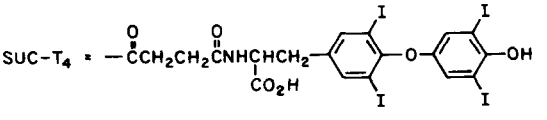


	R^1	R^2
1	H	H
2a	Tr	H
b	BOC	H
3a	Tr	NPE
b	Tr	PA
c	BOC	NPE
d	BOC	PA
4a	H	NPE
b	H	PA

	R^1	R^2
6a	PG'(PA)	NPE
b	PG'(PA)	PA
7	PG'	H
8	SUC	PA
9	SUC-T_4	PA
10	SUC-T_4	H

5 

SUC = $-CCH_2CH_2COH$



PE triester intermediates, required for ligand or analyte coupling are prepared from beta, gamma-dipalmitoyl-DL-alpha-phosphatidylethanolamine (1) in a three step reaction sequence. The primary amine is blocked with trityl or BOC groups using trityl chloride (4.6 eq.)/Et_3N (1.5 eq.) ($CHCl_3$; 55°, 6 hrs.) or BOC-ONB (1.2 eq.)/Et_3N(1.5 eq.) (50% aqueous dioxane, 55°, 3 hrs.) respectively to give 2a (46%)[20] and 2b (97%). These are converted to the p-nitrophenethyl phosphotriesters 3a (62%) and 3c (29%) using p-nitrophenethyl alcohol (4 eq.), 8-quinolinesulfonylchloride (1.5 eq.) and 3-nitro-1,2,4-triazole (3 eq.) (pyridine, 22°, 3 days)[21]. The corresponding p-bromophenyacyl phosphotriesters 3b (44%) and 3d (60%) are prepared by reaction of 2 with alpha, p-dibromoacetophenone (1.5 eq.)/Et_3N(1.0 eq.) (DMF/THF, 1:1 or 7:2, 22°, 20 hrs.). This is the first reported use of the phenacyl group for phosphate protection. Trityl and BOC groups are cleaved quantitatively with 80% HOAC (16 hrs.) or 30% TFA/CH_2Cl_2 (30 min.) respectively giving 4a (>90%) and 4b (>90%).

Penicillin G was converted to the more soluble p-bromophenacyl ester 5 (47%) using alpha, p-dibromoacetophenone (1.5 eq.)/ET_3N (1.0 eq.) (DMF/THF, 1:1, 5°, 4 hrs.)[22]. Reaction of 4a or 4b with 5 (1.5-10 eq.) ($CHCl_3$, 22°, 20 hrs.) gives the triester conjugates 6a (95%) and 6b (48%, yield not optimized). 6a was fully deprotected using 1.8-diazabicyclo-[5.4.0]undec-7-ene (DBU) (5 eq.) ($CHCl_3$, 22°, 20 hrs.) to give chromatographically pure 7 (78%) after silica gel PLC ($CHCl_3$/MeOH/$H_2$0, 65:25:4) to remove trace impurities. FAB mass spectral analysis indicated molecular ions at m/e 1049, 1071 and 1093 corresponding to the mono-, di- and tri- sodium complexes. Similar deprotection of 6b gave 7 in undetermined yield as characterized by TLC ($CHCl_3$/MeOH/$H_2$0, 65:25:4).

L-thyroxine (T_4) is indirectly coupled to PEA via an intermediate succinamide ligand in a two step reaction sequence. The phosphotriester 4b is succinoylated with succinic anhydride (1.2 eq.) (DMF/$CHCl_3$, 1:1, 55°, 3 hrs.) to afford the hemisuccinate 8 (44%). This is activated with DCC (1.0 eq.) (CH_2Cl_2,22°, 0.1 hrs.) and reacted with L-thyroxine (1.0 eq.) (equal volume of DMF, 22°, 4 hrs.) to give the succinamide linked phosphotriester-T_4 conjugate 9 (30%). Deprotection with DBU (9 eq.) ($CHCl_3$, 22°, 2 hrs.) and silica gel PLC ($CHCl_3$/MeOH/H_2O, 54:40:5) affords the target compound (10) (52%). Structure was verified by FAB mass spectroscopy which gave a single molecular ion for the free acid at m/e 1550.

References

1. Gregoriadis, G. in Liposomes in Biological Systems, p. 25, Gregoriadis, G. and Allison, A.C., ed., John Wiley and Sons, New York, 1980.

2. Alving, C.R. and Richards, R.L. in Liposomes, p. 209, Ostro, M.J., ed., Marcell Dekker, Inc., New York, 1983.

3. Kinsky, S.C., Biochim. Biophys. Acta, 265, 1 (1972).

4. Haxby, J.A., Kinsky, C.B. and Kinsky, S.C., Biochemistry, 61, 300 (1968).

5. Wei, R., Alving, C.R., Richards, R.L. and Copeland, E.S., J. Immunol. Methods, 9, 165 (1975).

6. Rosenqvist, E. and Vistnes, A.I., J. Immunol. Methods, 15, 147 (1977).

7. Chan, S.w., Tan, C.-T. and Hsia, J.C., J. Immunol. Methods, 21, 185 (1978).

8. Haga, M., Itagaki, H., Sugawara, S. and Okano, T., Biochem. Biophys. Res. Comm. 95, 187 (1980).

9. Tan, C.T., Chan, S.W. and Hsia, J.C., Meth. Enzymol., 74, 152 (1981).

10. Cole, F.X. U.S. Patent No. 4,342,826 (1982).

11. Braman, J.C., Broeze, R.J., Bowden, D.W., Myles, A., Fulton, T.R., Rising, M., Thurston, J., Cole, F.X. and Vovis, G.F., Bio/Technology, 2, 349 (1984).

12. Uemura, K. and Kinsky, S.C., Biochemistry, 4085, (1972).

13. Ryu, E.K., Ross, R.J., Matsushita, T, MacCoss, M., Hong, C.I. and West, C.R., J. Med. Chem., 25, 1322 (1982).

14. Hsia, J.C. and Tan, C.T., U.S. Patent NO. 4,235,792.

15. vanBoeckel, C.A.A. and vanBoom, J.H., Tetrahedron Letters, 21, 3705 (1980).

16. vanBoeckel, C.A.A., van der Marel, G.A., Westerduin, P. and vanBoom, J.H., Synthesis, 399 (1982).

17. Billimoria, J.D. and Lewis, K.O., J. Chem. Soc. (C), 1404 (1968).

18. Unless indicated otherwise, all products are isolated by silica gel PLC or short column chromatography using $CHCl_3$/MeOH mixtures or gradients. Key intermediates and all products have been characterized by I.R., NMR, mass spectral or elemental analysis.

19. Cole, F.X., Clough, K., Myles, A. and Law, S.-J., unpublished results.

20. Aneja, R., Chadha, J.S. and Knaggs, J.A., Biochim. Biophys. Acta, 187, 579 (1969).

21. Charubala, R. and Pfleiderer, W., Nucleic Acids Symposium Series No. 9, 161 (1981).

22. Bamberg, P., Ekstrom, B. and Sjoberg, B., Acta Chem. Scand., 21, 2210 (1967).

(Received in USA 30 September 1985)