Bioorganic & Medicinal Chemistry Letters, Vol.2, No.9, pp. 1007-1012, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

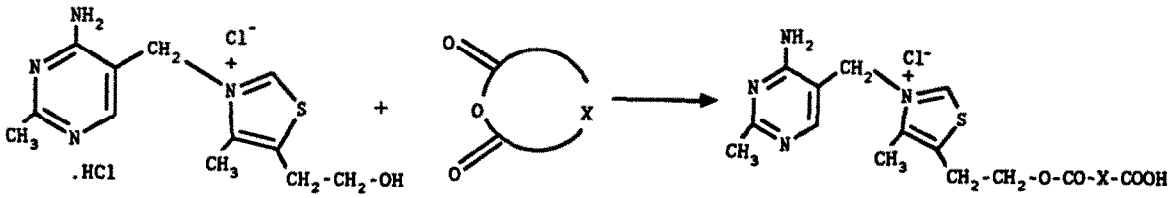
AN EFFICIENT METHOD FOR CONJUGATION OF THIAMINE TO PROTEINS

M. Jayamani and Philip S. Low
Chemistry Department, Purdue University, W. Lafayette, IN 47907

(*Received* 10 *April* 1992)

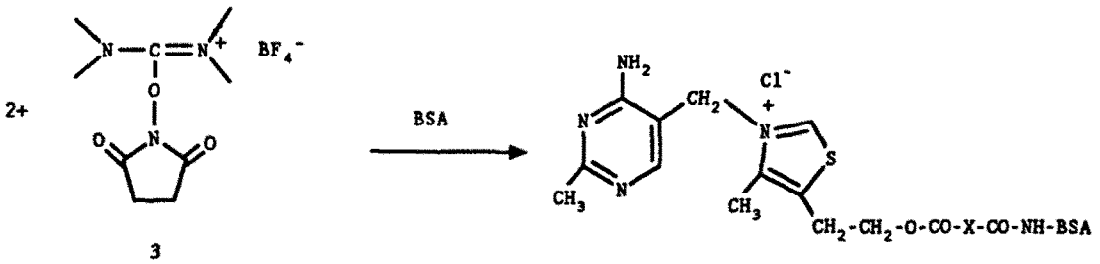
ABSTRACT: New derivatives of thiamine were prepared by reacting succinic, glutaric or maleic anhydride with the hydroxyl group of thiamine. The resulting free carboxyl group was then activated using N, N, N´,N´-tetramethyl-O-(-N-succinimidyl)uronium tetrafluoroborate[1] 3 and conjugated to proteins. Alternatively, proteins were reacted with 2-iminothiolane and the resulting sulfhydryl groups were then added across the double bond of thiamine monomaleiate 2c prepared by the above method.

Recently, we reported that vitamin receptor-mediated endocytosis can be exploited to deliver macromolecules nondestructively into living cells if the macromolecules are first covalently linked to folic acid[2a](a members of the vitamin B family) or biotin[2b]. This observation prompted us to explore thiamine[3], an essential coenzyme and a member of the vitamin B family, as a possible ligand to mediate the uptake of macromolecules into cells and tissues expressing cell surface thiamine receptors. The present paper describes new simple methods for conjugation of thiamine to proteins.



1 2

x in a = $-(CH_2)_2-$, b = $-(CH_2)_3-$, c = -CH=CH-.



3

Scheme - 1

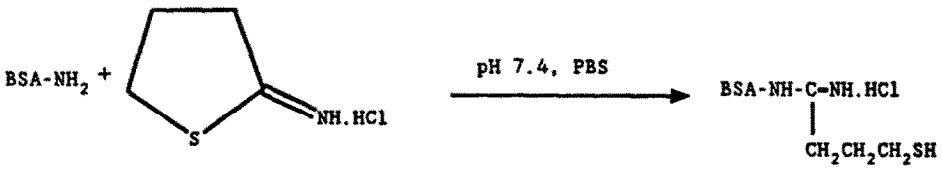
Our strategy(Scheme-1) was to open a cyclic anhydride 1 using the hydroxyl group of thiamine as a nucleophile. The derived product contains not only a free carboxyl at the open end which can be linked to a protein, but also the ester linkage to thiamine which could conceivably be readily hydrolyzed by esterases after entry of the protein into the cell. In addition, the long aliphatic spacer contributed by the anhydride should provide better separation of thiamine from the protein and allow improved recognition of the vitamin by its cell surface receptor.

Attempts to form thiamine monosuccinate 2a by direct heating(90°C) of thiamine chloride.HCl with succinic anhydride, or by heating(90°C) the same reaction components in pyridine did not yield any isolable product. However, when a suspension of thiamine chloride.HCl (3.37 g, 10 mmole) in pyridine (50 ml) was heated (80°C) with succinic anhydride (5 g, 50 mmole) and 4-(N,N-dimethyl)aminopyridine (1.22 g, 10 mmole), the product thiamine monosuccinate[4] 2a was obtained in excellent yield (3.2 g, 80%). The product was filtered, washed with acetone, and recrystallized from 95% ethanol (mp,180-5°C decom.)

Linkage to a protein was as follows: To a suspension of thiamine monosuccinate 2a (1.28 mg, 2.94 μmole) in DMSO (100 μL), N,N,N´,N´-tetramethyl-O-(-N-succinimidyl)uronium tetrafluoroborate 4 (0.88 mg, 2.94 μmole) and triethylamine (0.59 mg, 5.88 μmole) were added and stirred for 15 min. The above mixture was then added slowly to a solution of bovine serum albumin (BSA) (10 mg, 0.147 μmole) in phosphate buffered saline (PBS) (1 mL) pH 7. After 1h, the thiamine labeled BSA was separated from other components and the pH was changed to 5 by passing the mixture through a 1cm x 10cm Sephadex-G25 desalting column. BSA concentration was then estimated by the BCA method[5]. Thiamine attached to BSA was estimated[6] (3 thiamines per BSA) by oxidizing the conjugated thiamine to thiochrome and measuring the level of fluorescence at excitation 365 nm and emission 445 nm. For this purpose, a standard curve was constructed from the fluorescence of a known amount of thiamine mixed with the same amount of unmodified BSA sample. The low extent of BSA derivatization may have derived from the instability of the activated thiamine ester in aqueous solution or from the tendency of hydrophobic molecules to bind noncovalently to lipophilic sites on BSA.

The poor solubility of thiamine monosuccinate 2a in DMSO prompted us to look for a still better derivative. Thus, thiamine monoglutarate 2b was prepared in 85%(3.5 g) yield

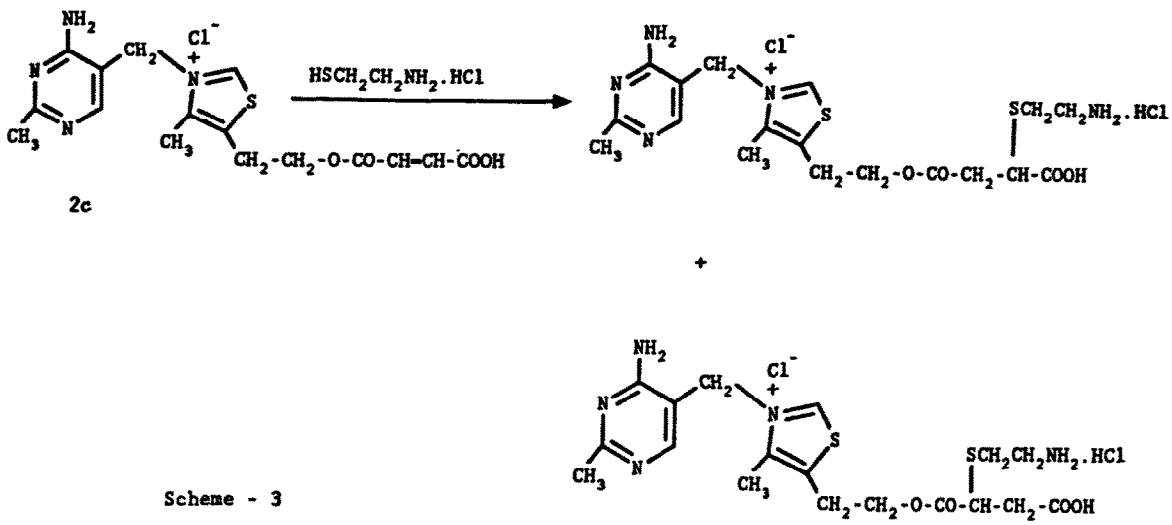
by stirring and heating (80°C) a suspension of thiamine chloride.HCl (3.37 g, 10 mmole) in pyridine (50 mL) with glutaric anhydride (5.7 g, 50 mmole). The product[7] 2b was purified (mp, 200°C decom.) as described for 2a. It may be worth mentioning that the presence of 4-(N,N-dimęthyl)aminopyridine led to decomposition of the product and glutaric anhydride. Thiamine monoglutarate 2b was activated and reacted with BSA as in 2a and the resulting thiamine content (4 thiamine per BSA) was estimated[6].



4

Scheme - 2

A large number of proteins have sulfhydryl groups and those lacking thiols can be easily modified to acquire them(Scheme-2). Such sulfhydryl groups can then be easily added to a reactive double bond[8]. To exploit this conjugation chemistry, thiamine monomaleiate[9] 2c was prepared in 83%(3.3 g) yield by heating (85°C) and stirring(3 hr) a mixture of thiamine chloride.HCl (3.37 g, 10 mmole) and maleic anhydride (4.9 g, 50 mmole) and the product was purified (mp 230-2°C decom.) as 2a. To test the proposed reaction (Scheme-3), thiamine monomaleiate 2c (0.4 g, 1 mmole) was reacted with 2-aminoethanethiol.HCl (0.114 g, 1 mmole) in 25% aqueous methanol (5 ml) at 25°C for 12 h. After methanol removal by rotary-evaporation and crystallization in 95% ethanol, the product 2 or 3-(2'-aminoethylthio) 4-(thiamine)monosuccinate 5[10] (mp 165-70°C decom.) was obtained quantitatively. However, no attempts were made to find the ratio of the positional isomers.



2c

5

Scheme - 3

BSA (10 mg, 0.145 μmole) in PBS (pH 8) was reacted with iminothiolane.HCl 4 (2.94 μmole) for 3 h at 25°C. Unreacted iminothiolane was removed and the pH was adjusted to 5 by passing the above reaction mixture through a 1 cm x 10 cm Sephadex-G25 desalting column. The derivatized BSA was treated immediately with thiamine monomaleiate 2c (1.17 mg, 2.94 μMole), and after 3 h the BSA was purified and the number of thiamines per BSA was estimated[6](3:1).

In conclusion, we have reported a simple method to attach thiamine to any protein, thus opening a path to a number of biological investigations. Details regarding thiamine-mediated delivery of macromolecules into living cells will be reported elsewhere.

Acknowledgment: We gratefully acknowledge a research grant from Eli Lilly.

References and Notes:

1.a. Bannwarth, W.; Schmidt, D.; Stallard, L. R.; Hornung, C.; Knorr, R. and Müller, F.; Helv. Chem. Acta. 1988, *71*, 2085.

b. Knorr, R.; Trzeciak, A.; Bannwarth, W. and Gillessen, D.; Tetrahedron lett. 1989, *30*, 1927.

2.a. Leamon, C. P. and Low, P. S.; Proc. Natl. Acad. Sci. USA. 1991, *88*, 5572.

b. Horn, M. A.; Heinstein, P. F. and Low. P. S.; Plant Phys. 1990, *93*, 1492.

3. Kluger, R.; Chem. Rev. 1987, *87*, 863.

4. For 2a. ^{1}H-NMR(200MHz,D_2O) δ (overlapping two s,6H & a m,4H)2.4-3.2, (t,2H)3.5-3.7, (t,2H)4.5-4.7, (s,2H)5.5, (s,1H)7.9, (s,1H)9.7. ^{13}C-NMR(200MHz, D20) δ 13.67, 24.38, 28.43, 28.43, 33.61, 53.09, 66.29, 108.33, 138.29, 146.49, 151.138, 165.8, 165.81, 167.85, 178.28, 182.71. IR(nujol) cm^{-1} 3280, 1745, 1660, 1595, 1560, 1210, 1155. U V(water) Emax.12238/237nm. CI.M^+ 365.1276 (Calcd. for $C_{16}H_{21}N_4O_4S^+$ 365.1284)

5. Smith, P. K.; Krohn, R.I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimato, E. K.; Coeke, N. M.; Olson, B. J. and Klenk, D. C.; Anal. Biochem., 1985, *76*, 150.

6. The United States Pharmacopeia xxth rev. United State Pharmacopeia Convention, Inc. Rockville, MD., 1980, p.928.

7. For 2b. ^{1}H-NMR(200MHz,D_2O) δ (q,2H)1.7-1.9, (overlapping two s,6H & a m, 4H)2.3-2.5, (t,2H) 3.3-3.4, (t,2H)4.2-4.3, (s,2H)5.5, (s,1H)7.9, (s,1H)9.6. ^{13}C-NMR(200MHz,D_2O) δ 13.67, 22.13, 23.59, 28.45, 35.44, 35.55, 52.8, 68.22, 108.85, 138.52, 146.53, 147.77, 157.96, 166.15, 166.36, 178.38, 180.81. IR(nujol)cm^{-1} 3200, 1730, 1650, 1600, 1525, 1210, 1155; U V(water) Emax. 15104/243nm, 12155/260nm. FAB.M^+ 379.1434 (calcd. for $C_{17}H_{23}N_4O_4S^+$ 379.1440)

8.a. Blatter, W. A.; Kuenzi, S. B.; Lambert, J. M. and Senter, P.D.; Biochemistry 1985, *24*, 1517.

b. Partis, M. D.; Griffiths, D. G. and Roberts, G. J.; Pro. Chem., 1983, *2*, 263.

9. For 2c. ^{1}H-NMR(200MHz,D_2O) δ (s,3H)2.48, (s,3H)2.56, (t,3H)3.3-3.4, (t,2H)4.4-4.5 (s,2H)5.5 (d,1H)6.1-6.2, (d,1H)6.5-6.5, (s,1H)7.9, (s,1H)9.6.^{13}C-NMR(200MHz,D_2O) δ 13.63, 23.52, 28.36, 52.77, 66.91, 108, 129.7, 136.1, 138.33, 146.7, 147.69, 157.98, 166.11, 166.76, 169.7, 173. IR(nujol)cm^{-1} 3210, 1715, 1745, 1655, 1655, 1645, 1605, 1595, 1515, 1155. U V(water)Emax. 11117/242nm, 10643/260nm. FAB.M^+ 363.1105 (calcd. for $C_{16}H_{19}N_4O_4S^+$ 363.1127)

10. For 5. ^{1}H-NMR(200MHz,D_2O) δ (s,3H)2.48, (s,3H)2.55, (m,4H)2.7-3, (m,4H)3.1-3.3, (m,1H)3.6-3.6, (t,2H)4.4-4.2, (s,2H)5.5, (s,1H)7.9, (s,1H)9.6.^{13}C-NMR(200MHz,D_2O) δ 13.74, 23.63, 28.47, 31.05, 41.13, 44.33, 52.82, 66.71, 108.82, 138, 146.2, 147.83, 157, 161.15, 166.36, 175.34, 178.44. IR(nujol)cm^{-1} 3200, 1720, 1730, 1650, 1605, 1305, 1150, U V(water) Emax. 10989/242nm, 12192/260nm. FAB.M^+440.1409 (calcd. for $C_{18}H_{26}N_5O_4S_2^+$ 440.1426)