SYNTHESIS OF 1,1'-CARBONYLDIIMIDAZOLE-*1-^{14}C* AND ITS USE IN PREPARING A METHOXY(POLYETHYLENE)GLYCOL SEMICARBAZIDE LINKER

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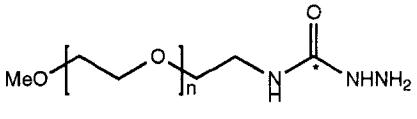
SUMMARY

Reaction of *N*-(trimethylsilyl)imidazole (**2**) with phosgene-*^{14}C* in toluene afforded 92 w/w % pure (^{1}H NMR) 1,1'-carbonyldiimidazole-*1-^{14}C* (**3**) in near quantitative yield. Activation of MPEG-amine **4** by reaction with **3** in dichloromethane afforded intermediate **5** in situ. Further reaction with hydrazine in toluene at 50°C and reprecipitation from 2-propanol afforded crude **1** (81%). A two-step chromatographic purification of this material followed by reprecipitation from 2-propanol gave MPEG-semicarbazide linker **1**. This material had a chemical purity estimated at 98%, a radiochemical purity of 95%, a specific activity of 7.7 μCi / mg (37.6 mCi / mmol) and a number-average molecular weight of 4885 (^{1}H NMR).

Key words: methoxy(polyethylene)glycol linker, 1,1'-carbonyldiimidazole, semicarbazide, carbon-14

INTRODUCTION

Covalent attachment of monomethoxy(polyethylene)glycols (MPEGs) and poly(ethylene)glycols (PEGs) to substrates of medicinal interest frequently yields physiologically active adducts whose pharmacokinetic parameters such as bioavailability, solubility, absorption, membrane binding or permeability and blood circulating half-life relative to the parent materials are improved.[1-16] Also, these adducts are usually less immunogenic and antigenic in vivo than the parent materials.[1] For these as well as other reasons, evaluation of a series of adducts obtained by reaction of various MPEG-linkers with a glycoprotein was undertaken.[17,18] From this work, it became necessary to prepare carbon-14-labeled MPEG-semicarbazide linker **1**, which is reported herein.



1

* indicates position of carbon-14 label

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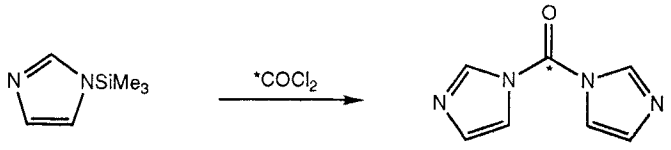
RESULTS AND DISCUSSION

Although several radiolabeled MPEG-linkers have been reported,[19-21] in no case was the label incorporated as in the present study. Introduction of the carbon-14 label required the use of 1,1'-carbonyldiimidazole-*1-14C* (**3**). Of reported methods of preparation of the unlabeled reagent,[22-34] the method shown in Scheme 1[25,30] was best suited for our purposes. Reaction of phosgene-*14C* with 2.03 equiv *N*-(trimethylsilyl)imidazole (**2**) at low temperature in toluene followed by slow warming of the mixture

Scheme 1



2 **3**

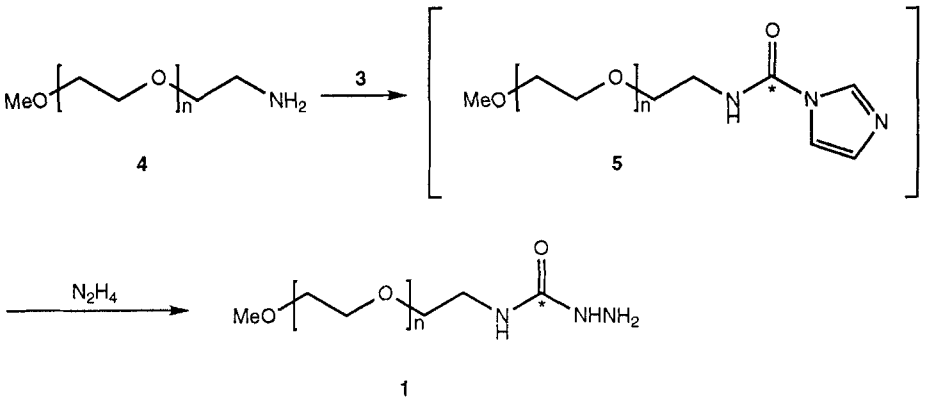
* indicates position of carbon-14 label

to ambient temperature and concentration in vacuo gave **3** in near quantitative yield. The ^{1}H NMR spectrum and melting point of **3** were in good agreement with data for the unlabeled material. The ^{1}H NMR spectrum showed contamination with imidazole (ca. 8 w/w %) and toluene (trace). Thus, a purity of 92 w/w % was assigned to **3**, which was used immediately in the next reaction.

Reaction of MPEG-amine **4** with **3** (2.5 equiv)[35] in CH_2Cl_2 for 2.5 h followed by an aqueous work up, drying and concentration in vacuo provided intermediate **5** (Scheme 2), which was used immediately without further purification. Dissolution of **5** in warm toluene (50°C), reaction with hydrazine (5 equiv) for 3 h, concentration in vacuo and reprecipitation from hot 2-propanol to remove imidazole (reaction by-product) gave crude MPEG-semicarbazide linker **1** as a colorless powder (81%).

Since reported methods of purification[1,5,7,8,13,15,20,36-42] and analysis[43-51] of related MPEGs were unsuccessful with carbon-14-labeled **1**, development of alternative methods was of critical importance. After much experimentation, both analytical and preparative reversed phase gradient HPLC methods were developed. Crude **1** was estimated to have a chemical purity of 39% and a radiochemical purity of

Scheme 2



* indicates position of carbon-14 label

44%. These low values were due, in part, to the starting MPEG-amine **4** being a multicomponent mixture (HPLC). A two-step chromatographic purification of a portion of crude **1** followed by reprecipitation from hot 2-propanol returned **1** (122 mg) as a colorless powder having an estimated chemical purity of 98%, a radiochemical purity of 95% and a specific activity of 7.7 μCi / mg. The observed number-average molecular weight, M_n, calculated according to equation 1 from integrals in the NMR spectrum,[51,52] was determined to be 4885. From this analysis, the number of repeating C_2H_4O units in the backbone of **1** was 108.

$$M_n = [(\text{integral backbone} - 2) / (\text{integral methoxy} / 3)] / 4 \text{ X } 44 + 133 \quad \text{(eq 1)}$$

EXPERIMENTAL

^{1}H NMR spectra were recorded on a Bruker AM360 spectrometer. Chemical shifts are expressed on the ∂ scale and reported downfield of tetramethylsilane (TMS) internal standard. The infrared spectrum (KBr pellet) of **1** was recorded on a Perkin-Elmer model 283 infrared spectrophotometer. The melting point of **3** (uncorrected) was taken on a Thomas-Hoover capillary melting point apparatus. Radioactivity measurements were performed using a Beckman model LS6000LL liquid scintillation counter. The analytical HPLC data were acquired on a Waters Millenium 2010 HPLC system consisting of a model 600E system controller, a model 996 diode array detector and a model 717 WISP injector. Absorption was

monitored at a wavelength of 192 nm with 1.0 AUFS using a Zorbax cyano column (5 µm packing, 4.6 x 150 mm) purchased from MAC-MOD (Chadds Ford, PA). Radiochemical detection was accomplished using a Radiomatic model A250 flow detector attached downstream from a Hewlett Packard 1090 liquid chromatograph equipped with Chemstation software and the Zorbax cyano column (192 nm). Preparative chromatography was performed on a Waters HPLC system consisting of a model 600E system controller, a model 486 variable wavelength detector and a Rheodyne 7125 manual injector equipped with a 2 mL sample loop. Absorption was monitored at a wavelength of 192 nm with 1.0 AUFS using a Zorbax cyano column (7 µm packing, 21.2 x 250 mm) purchased from MAC-MOD (Chadds Ford, PA). Phosgene-^{14}C (150 mCi) was obtained from DuPont NEN Research Products (Boston, MA) and used as received. The MPEG-amine **4** (average molecular weight of 5000) was purchased from Shearwater Polymers, Inc. (Huntsville, AL) and used as received. Anhydrous methylene chloride (CH_2Cl_2), hydrazine (98%) and *N*-(trimethylsilyl)imidazole (97%) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received. Anhydrous magnesium sulfate ($MgSO_4$), sodium perchlorate (HPLC grade), concentrated aqueous perchloric acid (ACS grade), toluene (ACS grade) and 2-propanol (ACS grade) were purchased from Fisher Scientific Co. (Malvern, PA) and used as received. Elemental analysis of chromatographically purified, unlabeled MPEG-semicarbazide linker **1** was performed by Robertson Microlit Laboratories, Inc. (Madison, NJ) under optimized conditions (±0.1%).

1,1'-Carbonyldiimidazole-*1*-^{14}C (3)

An oven-dried 50 mL flask and stir bar were cooled to ambient temperature under a stream of dry nitrogen. The flask was charged with toluene (14 mL) and 0.77 mL (5.25 mmol, 2.03 equiv) *N*-(trimethylsilyl)imidazole (**2**). The flask was placed onto a vacuum line, and the solution degassed after three freeze / thaw cycles. After placing the flask under vacuum and cooling the solution to just above its freezing point (ca. -90°C), the phosgene-^{14}C was vacuum transferred into the flask with stirring. The resulting colorless slurry was stirred in vacuo (closed system) while warming to ambient temperature. After 3.25 h, volatiles were removed by careful evaporation at ambient temperature on a rotary evaporator. Further drying in vacuo at ambient temperature to constant weight gave 443 mg (>100%) 1,1'-carbonyldiimidazole-*1*-^{14}C (**3**) as a colorless solid: mp (observed) 111-115°C; mp (reported for unlabeled **3**)[22] 115.5-116°C; ^1H NMR (CD_2Cl_2) ∂7.14 (m, 2H), 7.46 (m, 2H), 8.10 (br s, 2H). The NMR spectrum also showed contamination by ca. 8 w/w % imidazole and toluene (trace). Thus, a purity of 92 w/w % was assigned to **3**. This material was used immediately in the next reaction.

MPEG-Semicarbazide linker 1

An oven-dried 50 mL two-neck flask and stir bar were cooled to ambient temperature under a stream of dry nitrogen. The flask was charged with a solution of 221.5 mg (1.25 mmol based on 92 w/w % purity, 2.5 equiv)[35] **3** in dry CH_2Cl_2 (10 mL). Under nitrogen, a viscous solution of 2.51 g (0.50 mmol, 1.0 equiv) MPEG-amine **4** in CH_2Cl_2 (7 mL) was added with good stirring. The addition funnel was rinsed forward with CH_2Cl_2 (2 x 3 mL). After stirring for 2.5 h at ambient temperature, the clear light yellow solution containing **5** was diluted with CH_2Cl_2 (17 mL), washed with distilled water (12 mL), dried for 15 min over anhydrous $MgSO_4$, filtered and concentrated in vacuo for 1 h. Crude **5** was dissolved at 50°C in toluene (13 mL) under nitrogen with good stirring. After 5-10 min, 79 µL (2.5 mmol, 5.0 equiv) hydrazine was added dropwise by microsyringe. After an additional 3 h, the clear, colorless solution was cooled to ambient temperature and concentrated to constant weight in vacuo to afford 2.75 g (>100%) crude **1** as a colorless powder. Reprecipitation from hot 2-propanol (58 mL), filtration, washing the filtercake with 2-propanol (2 x 5 mL) and drying in vacuo to constant weight gave 2.05 g (81%) **1** as a colorless powder. HPLC analysis gave an estimated chemical purity of 39% and a radiochemical purity of 44%. HPLC samples were prepared in 25 / 75 MeCN - water (v/v, 4.3 mg / mL). It should be noted that prior to the actual analyses, the column was conditioned by running a blank in triplicate. During this conditioning, some sodium perchlorate was bound to the column. Without this conditioning, no separation occurred. Successful separation of components was achieved during the salt gradient portion of the elution; use of aqueous acetonitrile mobile phases gave poorly resolved broad peaks. It was also found that the most difficult part of the analysis was determining the starting ratio of MeCN / water, as the ratio changed after prolonged column use, or vigorous cleaning or base washing of the column. During two months of use, the initial % MeCN increased from 24 to 40%. Once an acceptable separation was achieved, repetitive injections over several days did not appreciably change chromatographic performance. The gradient program used for these analyses is shown in Table 1. All gradient changes were linear. The buffer used was 0.5*M* sodium perchlorate adjusted to pH 2.5 with perchloric acid. The product (**1**) eluted at a retention time t_R = 5.0 min.

Table 1

Time (min)	0	2	15	17	19	20	21	24
% MeCN	25	25	27	27	75	75	25	25
% Water	75	75	0	0	0	0	75	75
% Buffer	0	0	73	73	25	25	0	0
Flow (ml /min)	1	1	1.5	1.5	1.5	1.5	1	1

A portion of crude **1** (ca. 940 mg) was subjected to a two-step purification using the preparative Zorbax cyano column. As with the analytical HPLC system, the column was first conditioned by running a blank in

triplicate. Next, a sample of unlabeled **1** was injected to verify that the column was ready for use. The sample was dissolved in 28 / 72 MeCN - pH 2.5, 0.5*M* sodium perchlorate (v/v; 160 mg / mL) and 0.5 mL of this solution was injected per run. The HPLC gradient program used is shown in Table 2. An initial flow rate of 4 mL / min was used to allow for the back pressure increase on injection due to the high viscosity of the sample solution. The abruptness of changes in the gradient program was done to reduce the run time while maintaining acceptable resolution of components. All gradient changes were linear. The buffer used was 0.5*M* sodium perchlorate, adjusted to pH 2.5 with perchloric acid.

Table 2

Time (min)	0	1	5	15	16	17	18	21	22	25
% MeCN	34	34	34	35	35	35	75	75	34	34
% Water	66	66	66	32	0	0	0	0	66	66
% Buffer	0	0	0	33	65	65	25	25	0	0
Flow (mL/min)	4	15	15	15	15	15	15	15	15	15

The major peak eluting from 6.4 - 9.0 min was collected, the solvent was evaporated at 30°C (bath temperature) in vacuo to a small volume (<5 mL) and diluted with water (ca. 10 mL). This clear, colorless aqueous solution containing **1** was rechromatographed through the preparative Zorbax cyano column according to the HPLC gradient program listed in Table 3. Again, an initial flow rate of 4 mL / min was used to allow for the back pressure increase on injection due to the high viscosity of the sample solution. The abruptness of changes in the gradient program was again done to reduce the run time while maintaining acceptable resolution of components. All gradient changes were linear. A total of 2 mL of solution was

Table 3

Time (min)	0	1	4	6	8	18	20	22	23	27
% MeCN	0	0	0	32	33	45	100	100	0	0
% Water	0	100	100	68	67	55	0	0	0	0
% 5% HOAc	100	0	0	0	0	0	0	0	100	100
Flow (mL/min)	4	12	12	15	15	15	15	15	15	15

injected onto the column per run. Subsequent injections were made once the column equilibrated to initial conditions (run time = 30 min per injection). The peak eluting from 13.2 - 17 min was collected and the solution was concentrated to constant weight in vacuo to yield 169 mg (18% weight recovery) **1** as a colorless powder. The ^{1}H NMR spectrum (CD_2Cl_2) of this material showed contamination by an impurity(ies) containing a (cyano)propylsilyl residue, probably introduced as a leachate from the preparative Zorbax cyano HPLC column during the purification sequence. Reprecipitation of this material from hot 2-propanol gave 122 mg (13% weight recovery) MPEG-semicarbazide linker **1** as a colorless powder: IR (KBr) 1610 cm^{-1}; ^{1}H NMR (CD_2Cl_2) ∂3.33 (s, 3H, OCH_3), 3.44 (m, 2H, $OCH_2C\underline{H}_2NHCONHNH2$), 3.60 (s, 436H, $CH_3O(C\underline{H}_2C\underline{H}_2O)_{108}C\underline{H}_2$-). HPLC analysis indicated a chemical purity estimated at 98% and

a radiochemical purity of 95%. The specific activity was determined to be 7.7 μCi / mg (37.6 mCi / mmol based on a number-average molecular weight of 4885; NMR, see equation 1).

A sample of unlabeled **1**, isolated from an identical preparation sequence, gave the following elemental analysis results under optimum combustion conditions: C, 53.60; H, 9.30; N, 0.77. Based on the NMR derived number-average molecular weight M_n = 4885 (n = 108 in **1**), calculated %'s for $C_{220}H_{443}N_3O_{110}$ were: C, 54.03; H, 9.13; N, 0.86.

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REFERENCES AND NOTES

1. Harris J.M., Ed., "Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications," Plenum: New York, 1992 and references cited therein.
2. Greenwald R.B., Pendri A., Bolikal D. and Gilbert C.W. - Bioorg. Med. Chem. Lett. 4: 2465 (1994).
3. Greenwald R.B. and Bolikal D. - PCT Intl. Appl. WO9420453 (1993)(Derwent 94-302903/37).
4. Greenwald R.B. and Shorr R.G.L. - PCT Intl. Appl. WO9420089 (1993)(Derwent 94-302653/37).
5. Ladd D.L. and Snow R.A. - Anal. Biochem. 210: 258 (1993).
6. Ljunger G., Adlercreutz P. and Matiasson B. - Biocatalysis 7: 279 (1993).
7. Woghiren C., Sharma B. and Stein S. - Bioconjugate Chem. 4: 314 (1993).
8. Ikeda Y., Kai Y. and Ono K. - US 5183660 (1993)(Chem. Abst. 116: 231339x).
9. Desai N.P., Soon-Shiong P. and Sandford P.A. - PCT Int. Appl. WO9324476 (1992)(Chem. Abst. 120: 144136t).
10. Roseng L., Tolleshaug H. and Berg T. - J. Biol. Chem. 267: 22987 (1992).
11. Atassi M.Z., Ruan K.H., Jinnai K., Oshima M. and Ashizawa T. - Proc. Natl. Acad. Sci. U.S.A. 89: 5852 (1992).
12. Allen T.M., Hansen C., Martin F., Redemann C. and Yau-Young A. - Biochim. Biophys. Acta 1066: 29 (1991).
13. Sartore L., Caliceti P., Schiavon O. and Veronese F.M. - Appl. Biochem. Biotechnol. 27: 45 (1991).

14. Veronese F.M., Sartore L., Orsolini P. and Deghengi R. - PCT Int. Appl. WO9101758 (1991)(Chem. Abst. 115: 166605u).

15. Sartore L., Caliceti P., Schiavon O., Monfardini C. and Veronese F.M. - Appl. Biochem. Biotechnol. 31: 213 (1991).

16. Atassi M.Z. and Manshouri T. - J. Protein Chem. 10: 623 (1991).

17. Wright D.E. - European Patent Appl. 539167-A2 (1993) (Chem. Abst. 119: 56139v).

18. Wright D.E. - European Patent Appl. 605963-A2 (1994) (Derwent 94-219645/27).

19. Abuchowski A., Van Es T., Palczuk N.C. and Davis F.F. - J. Biol. Chem. 252: 3578 (1977).

20. Larwood D.J. and Szoka F.C. - J. Labelled Cpds. and Radiopharm. 21: 603 (1984).

21. Schiavon O., Caliceti P., Sartore L. and Veronese F.M. - Il Farmaco 46: 967 (1991).

22. Staab H.A. - Liebigs Ann. Chem. 609: 75 (1957).

23. Anderson G.W. and Paul R. - J. Am. Chem. Soc. 80: 4423 (1958).

24. Staab H.A. - Ger 1033210 (1958)(Chem. Abst. 54: 14272h).

25. Birkofer L., Richter P. and Ritter A. - Chem. Ber. 93: 2804 (1960).

26. Paul R. and Anderson G.W. - J. Am. Chem. Soc. 82: 4596 (1960).

27. Birkofer L., Ritter A. and Richter P. - Ger 1136342 (1962)(Chem. Abst. 58: 5724a).

28. Hecht R. and Rüchardt C. - Chem. Ber. 96: 1281 (1963).

29. Staab H.A. and Wendel K. - Chem. Ber. 96: 3374 (1963).

30. Walter W. and Radke M. - Liebigs Ann. Chem. 1756 (1979).

31. Sheludyakov V.D., Sheludyakova S.V., Kuznetsova M.G., Silkina N.N. and Mironov V.F. - J. Gen. Chem. USSR (Engl. Trans.) 50: 705 (1980).

32. Kiso Y. and Hayashi T. - JP 02207073 (1990) (Chem. Abst. 114: 62096s).

33. Kiso Y. and Hayashi T. - JP 02207074 (1990) (Chem. Abst. 114: 6510k).

34. Novy P.M. US 4965366 (1990)(Chem. Abst. 114: 43167z).

35. The reaction stoichiometry was not optimized.

36. Suzuki T. and Tomono T. - J. Poly. Sci. 22: 2829 (1984).

37. Veronese F.M., Largajolli R., Boccù E., Benassi C.A. and Schiavon O. - Appl. Biochem. Biotech. 11: 141 (1985).

38. Tondelli L., Laus M., Angeloni A.S. and Ferruti P. - J. Controlled Rel. 1: 251 (1985).

39. Iwasaki K., Iwashita Y. and Okami T. - US 4670417 (1987).

40. Leonard M. and Dellacherie E. - Makromol. Chem. 189: 1809 (1988).

41. Loccufier J., Crommen J., Vandorpe J. and Schacht E. - Makromol. Chem., Rapid Commun. 12:159 (1991).

42. Cuscurida M. and Faske A.J. - US 4994627 (1991).

43. Turner L.P., McCullough D. and Jackewitz A. - J. Am. Oil Chem. Soc. 53: 691 (1976).

44. Fukano K., Komiya K., Sasaki H. and Hashimoto T. - J. Chromatogr. 166: 47 (1978).

45. Sims G.E.C. and Snape T.J. - Anal. Biochem. 107: 60 (1980).

46. Van Der Wal S. and Snyder L.R. - J. Chromatogr. 255: 463 (1983).

47. De Vos R. and Goethals E.J. - Polymer Bulletin 15: 547 (1986).

48. Brown R.S., Weil D.A. and Wilkins C.L. - Macromolecules 19: 1255 (1986).

49. Escott R.E.A. and Mortimer N. - J. Chromatogr. 553: 423 (1991).

50. Seraglia R., Traldi P., Mendichi R., Sartore L., Schiavon O. and Veronese F.M. - Anal. Chim. Acta 262: 277 (1992).

51. Selisko B., Delgado C., Fisher D. and Ehwald R. - J. Chromatogr. 641: 71 (1993).

52. Dust J.M., Fang Z. and Harris J.M. - Macromolecules 23: 3742 (1990).