SYNTHESIS Of IODINE-125 LABELED PHORBOL ESTER--A USEFUL GAMMA-EMITTING ANALOG OF 12-O-TETRADECANOYLPHORBOL-13-ACETATE

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SUMMARY

Iodine-125 labeled analog of 12-O-tetradecanoylphorbol-13-acetate (TPA) was obtained by heating the iodine substituted analog of TPA in acetone with sodium [^{125}I] iodide. The iodine substituted analog of TPA, 12-O-(12'-iodododecanoyl)phorbol-13-acetate (IPA), was synthesized from phorbol by a three step reaction sequence. The structure of IPA was confirmed by high resolution proton magnetic resonance, fast atom bombardment mass spectrometry (FAB) and chemical ionization mass spectrometry (CIMS).

The [^{125}I]-IPA was purified by normal phase flash column chromatography. The product had 99% radiochemical purity and specific activity of Ca. 2.7Ci/mmol.

Key Words: Radioiodination, 12-O-Tetradecanoylphorbol-13-acetate, 12-O-(12'-Iodododecanoyl)phorbol-13-acetate, Phorbol esters, Phorbol esters receptors, Chemical carcinogens.

INTRODUCTION

Phorbol esters are a class of compounds which exhibit a variety of biological and biochemical effects in vivo and in vitro (1,2). One of these esters, 12-O-tetradecanoylphorbol-13-acetate (TPA, Fig. 1) is a potent tumor promoter in the two stage mouse skin model of carcinogenesis (3,4). Specific binding sites or receptors for the phorbol esters have been identified in a variety of cells and tissues (4-6). The agreement between the binding activity and potency for inducing biological responses among the various phorbol esters

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indicates that these receptors mediate the phorbol ester responses (6). Recent studies also indicate that multiple binding sites for phorbol esters exist in some tissues (5,7). The high affinity major binding sites are identical with the calcium and phospholipid dependent protein kinase (8,9) (PKC). It is generally believed that the phorbol esters induce their diverse biological effects by reversibly binding with PKC and activating this important enzyme. So far most of the binding studies have been carried out with the commercially available tritium labeled compounds 20-[^{3}H]-12-O-tetradecanoylphorbol-13-acetate (^{3}H-TPA) or 20-[^{3}H]-phorbol-12,13-dibutyrate (^{3}H-PDBu) as the specific ligands (4). Phorbol esters labeled with a fluorescent marker such as dansyl TPA have also been used for the identification of specific binding sites (10-12).

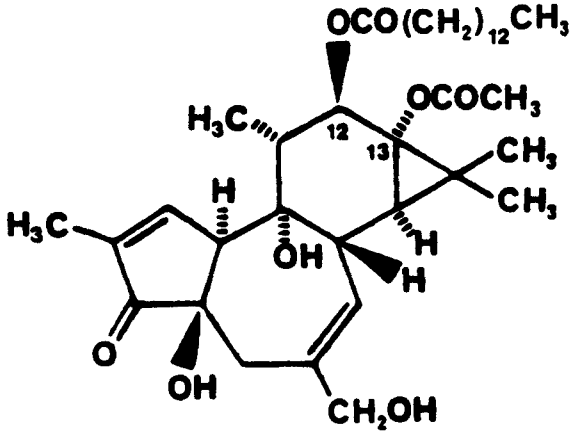


Figure 1. 12-O-Tetradecanoylphorbol-13-acetate.

In the present communication we describe the synthesis of an iodine substituted analog of TPA and the preparation of ^{125}I-labeled compound. The iodo-analog was found to modulate the cellular differentiation in human U-937 monocyte cells (13,14), one of the several cellular responses of phorbol esters. The biological activity of this analog appears to be qualitatively and quantitatively similar to that of TPA. The synthesized labeled compound has the unique advantage over other commercially available compounds of having a gamma emitting isotope in the long-chain fatty acid moiety of the molecule.

MATERIALS AND METHODS

General

Sodium [^{125}I] iodide was purchased from New England Nuclear (Boston, MA, U.S.A.). Precautions for the safe handling of ^{125}I recommended by the Department of Environmental Health and Safety, University of Minneota were followed. Radioiodination reaction was conducted in a well-ventilated, lead-shielded (3 mm thick) hood. Radioactive spots on TLC plates were located with a GM gamma counter. Iodine-125 radioactivity was measured with a Beckman model Gamma 5500 counter (Beckman Instruments, Inc., Irvine, CA 92713, U.S.A.). The solvents were evaporated under a slow stream of nitrogen at room temperature or on a hot water bath depending upon the boiling point of the solvent. Vapors from the evaporated solvents were vented into a sodium thiosulfate solution to trap any volatile iodine. Activated charcoal traps (NEN, Boston, MA, U.S.A.) were used to vent the air space before vials and containers were opened or radioactive solutions were transferred. All throughout this work closed systems were maintained.

Melting points were determined on a Thomas-Hoover melting point apparatus and are not corrected. Infrared (IR) spectra were obtained on a Perkin-Elmer 281 IR Spectrophotometer. Ultraviolet (UV) measurements were obtained on a Beckman model DU-8 spectrophotometer. Proton NMR spectra were determined at ambient temperature on a JEOL FX90 MHz or Nicollet 300 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained on a Finnigan 4000 instrument equipped to measure both positive and negative ions. Isobutane was used as the ionization gas. Fast atom bombardment (FAB) mass spectra were obtained on a VG7070EHF 11-250 data system instrument using the magic bullet matrix (16). Elemental analyses were performed by M-H-W laboratories, Phoenix, AZ and the results were within $\pm$ 0.4% of the theoretical values.

Thin layer chromatographic analyses were performed on silica gel GF or GHLF plates (250 micron, Analtech). The TLC plates were visualized under shortwave ultraviolet light followed either by iodine vapors development or by spraying with a vanillin-sulfuric acid-absolute ethanol (3:0.5:100) spray reagent and

heating over a hot plate at 120°C. Flash chromatographic purifications were accomplished using silica gel 60, 40-63 micron (230-400 mesh), EM Reagents (Merck, Darmstadt, F.R.G.) (15). The system was composed of a glass column (47.5x10 mm.I.D. or 47.5x20 mm.I.D.) and a flow control valve clamped at the top (Ace Glass, Inc., Vineland, NJ 08360, U.S.A.).

Croton oil was purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. and was stored at 4°C before use. Cyclododecanone, metachloroperbenzoic acid (MCPBA), hydroiodic acid, 1,3-dicyclohexyl carbodiimide (DCC), and 4-(N,N-dimethylamino)pyridine (DMAP) were obtained commercially (Aldrich Chemical Company, Milwaukee, WI, U.S.A.). All other chemicals used in this work were of reagent grade. The perchloric acid/methanol solution was prepared by diluting 70% perchloric acid (0.14 mL) with methanol (100 mL). Phosphate buffer (pH = 7.0) was prepared by mixing potassium dihydrogen phosphate 1/15 molar solution (41.3 mL) with disodium hydrogen phosphate 1/15 molar solution (58.7 mL). Unless otherwise indicated, solvents were dried before use.

Phorbol-13,20-diacetate (2):

Phorbol (1) (1 g, 2.44 mmol) was dissolved in a mixture of dry tetrahydrofuran (20mL) and dry dichloromethane (20 mL) and cooled in an ice bath. A solution of triethylamine (8 mL) in dichloromethane (15 mL) was added followed by a solution of acetic anhydride (6-7 mL) in tetrahydrofuran (10 mL). The reaction mixture was stirred vigorously under nitrogen atmosphere for 24 hrs. Water (20-30 mL) was added and the solution was adjusted to pH 7.0 by the dropwise addition of 2N sulfuric acid solution. The aqueous layer was extracted with dichloromethane (3x50 mL). The combined organic extracts was washed with pH = 7.0 phosphate buffer (25 mL) and dried over magnesium sulfate. The solution was filtered and the solvent was removed under reduced pressure. The thick oily residue was mixed with boiling ether and stored at 0°C for 48 hrs to give II as a white crystalline material (0.84 g, yield 70%); m.p. 183°C (Lit. 185°C) (18,23); R_f = 0.33 (dichlormethane-acetone, 6:1); ^{1}HNMR ($CDCl_3$-D_2O): δ 1.04(d, J = 5.6 Hz, 1H), 1.24(s, 9H), 1.79 (s, 3H), 1.9 (m, 1H), 2.06 and 2.1 (s, 6H), 2.4 (q, 2H), 3.1 (bm, 1H), 3.2 (bm, 1H), 3.98 (d, J = 10 Hz, 1H), 4.46 (S, 2H), 5.67 (m, H) and 7.58 (bs, 1H) ppm; CIMS: m/z 431 $(M+H-H_2O)^+$, 371 (M+H-

H_2O-$CH_3COOH)^+$, 353, 311; IR (KBr), ν_{max} (cm^{-1}) 3550, 3400, 2900, 1740, 1720, 1695, 1660; UV (methanol): λ_{max}nm(ϵ), 211 (8800), 258 (4000). Anal for $C_{24}H_{32}O_8.1.5\ H_2O$, Calculated: C,60.62; H,7.42. Found: C, 60.67; H, 7.45.

12-Iodododecanoic acid (8):

Cyclododecanone (6) (1g, 5.5 mmoles) and metachloroperbenzoic acid (2g, 10 mmoles) were dissolved in dry chloroform (10 mL). The solution was heated under Reflux for 48 hrs. The reaction mixture was cooled in an ice-water bath. The unreacted metachloroperbenzoic acid was filtered and the filtrate was evaporated to dryness. The residue left was digested with diethyl ether (50 mL). The ethereal extract was washed with 10% aqueous sodium sulfite solution (20 mL), 10% aqueous sodium carbonate solution (3x20 mL) and finally with brine solution (20 mL). The organic layer was dried with magnesium sulfate and concentrated in vacuo to give crude dodecanolide 7 (0.86 g, yield 80%). R_f = 0.67 (chloroform, silica gel).

Crude dodecanolide (7) (0.86 g, 4.3 mmoles) was dissolved in a mixture of glacial acetic acid (1.5 g) and hydroiodic acid (2.5 g). The mixture was maintained at 100°C for 2 hrs. The reaction mixture was cooled to room temperature and was poured to 10% aqueous sodium thiosulfate solution (50 mL). The aqueous layer was extracted with chloroform (3x25 mL). The combined organic extracts was dried over magnesium sulfate. After concentration of the organic extracts, the crude yellow colored oily residue was purified by flash column chromatography (20 g of silica gel 60 equilibrated with hexane-ethyl acetate-acetic acid, 80:20:1). Crystallization from petroleum ether provided pure 8 (1 g, yield 70%), mp = 61°C (Lit. 62.5°C[19]); R_f = 0.33 (dichloromethane-acetone-acetic acid, 90:10:1); R_f = 0.37 (ether-petroleum ether-acetic acid, 70:30:1); ^1H NMR ($CDCl_3$-D_2O): δ 3.17 (t, J = 7 Hz, 2H), 2.34 (t, J = 7 Hz, 2H), 1.72 (bm, 2H) and 1.27 (bs, 16H) ppm, COOH exchanged; IR (KBr): ν_{max} (cm^{-1}) 3000, 2900, 1690.

12-O-(12-Iodododecanoyl)phorbol-13,20-diacetate (3):

A mixture of 12-iodododecanoic acid (8) (140 mg, 0.4 mmol), phorbol-13,20-diacetate (2) (47 mg, 0.1 mmol), DMAP (6.5 mg, 0.05 mmol) in dry dichloromethane (1 mL) was cooled to -5°C in an ice/salt bath. DCC (43 mg, 0.2 mmol) was added

and the mixture was stirred under nitrogen atmosphere at -5°C for 1 hr and at room temperature for an additional 24-48 hrs. The progress of the reaction was monitored by TLC on silica gel plates using dichloromethane-acetone (6:1) as solvent. After the reaction was complete, the reaction mixture was concentrated in vacuo. The residue was mixed with ether (1 mL) and the ether extract was filtered to remove undissolved dicyclohexyl urea. The filtrate was mixed with pH = 7.0 phosphate buffer (10 mL) and ethyl acetate (10 mL). The aqueous phase was separated and extracted with ethyl acetate (2x10 mL). The organic solvent extracts were combined and dried over anhydrous magnesium sulfate. The dry extract was filtered and the filtrate was concentrated in vacuo. The crude resinous material obtained was purified by flash chromatography on silica gel (20 g in ether-petroleum ether 15:10) to give 3 (79 mg, yield 75%); R_f = 0.28 (ether-petroleum ether, 2:1); ^{1}H NMR ($COCl_3$-D_2O): δ 0.89 (d, J = 6.8 Hz, 3H), 1.08 (d, J = 5.14 Hz, 1H), 1.26 (s, 6H), 1.3 (bs, 20H), 1.8 (m, 3H), 2.06 and 2.1 (s, 6H), 2.24 (m, 1H), 2.48 (q, AB pattern, 2H), 3.2 (t, 3H), 4.46 (s, 2H), 5.44 (d, J = 10.3 Hz, 1H), 5.68 (m, 1H) and 7.56 (bs, 1H) ppm; FAB/MS, m/z 911 $(M+DTT+H)^+$, 779 $(M+Na)^+$ and 739 $(M+H-H_2O)^+$.

12-O-(12-Iodododecanoyl)phorbol-13-acetate (IPA, 4):

Compound 3 (60 mg, 0.08 mmol) was dissolved in perchloric acid solution in methanol (10 mL). The reaction mixture was purged with nitrogen, protected from light and stirred at room temperature for 24 hrs. A 0.5 M sodium methoxide solution was then added dropwise to adjust the pH to 6.0. The solution was concentrated to a small volume and added to pH 7.0 phosphate buffer (10 ml) and extracted with ethyl acetate (3x10 ml). The combined organic extracts were dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel (20 g in dichloromethane-acetone 5:1). Fractions containing the major component with R_f = 0.26 (toluene-ethylacetate 2:1) were combined and concentrated to yield 4 as a glassy resinous material (56 mg, yield 85%); ^{1}H NMR ($CDCl_3$-D_2O): δ 0.87 (d, J = 6.8 Hz, 3H), 1.07 (d, J = 5.14 Hz, 1H), 1.19-1.26 (bs, 26H), 1.76 (m, 3H), 2.07 (s, 3H), 2.2 (m, 1H), 2.5 (q, AB pattern, 2H), 3.2 (t, 3H), 4.0 (q, AB pattern, 2H), 5.4 (d, J = 10.3 Hz, 1H), 5.66 (m, 1H) and 7.57 (bs, 1H) ppm;

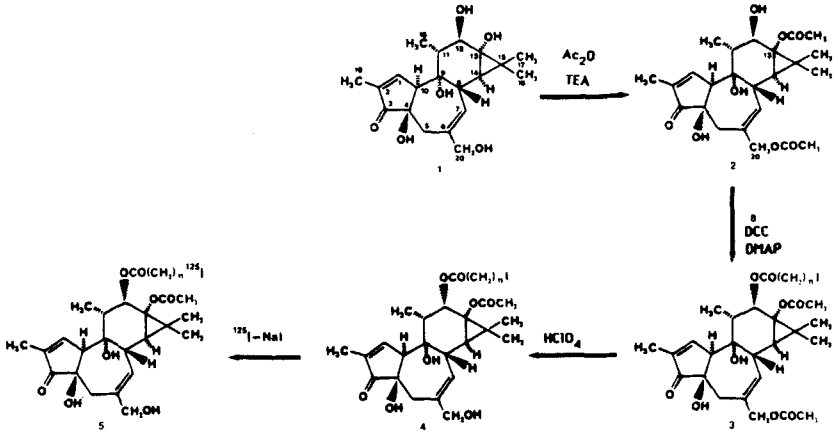
CIMS, m/z 371 $(M-I(CH_2)_{11}COO-H_2O)^+$, 311, 327 $(I(CH_2)_{11}COOH)^+$, 199; FAB/MS, m/z 737 $(M+Na)^+$, 389 $(M-I(CH_2)_{11}COO)^+$, 329, 311.

[^{125}I] 12-O-(12-iodododecanoyl)phorbol-13-acetate (5):

A solution of Na^{125}I (5 mCi, 17 Ci/mg, low pH and high concentration) in water (14 μL) was transferred into a sealed two-necked round bottom flask. Acetonitrile (100 μL) was injected into the reaction vessel and water was removed under a slow stream of nitrogen. A solution of IPA 4 (100 μg, 0.14 μmol) in dry acetone (100 μL) was added. The flask was purged with nitrogen, sealed tightly and heated over a water bath at 55°C for 4 hours. At the end of this period, ethyl acetate (10 mL) was added to the mixture. The ethyl acetate extract was washed with 0.1 N sodium thiosulfate solution (1x5 mL) and then with water (2x5 mL). The organic extract was dried over magnesium sulfate and filtered. The filtrate was concentrated under a stream of nitrogen. The crude residue was purified by flash chromatography on silica gel with dichloromethane/acetone (5:1, v/v) as the mobile phase. The eluant was collected in 5 mL fractions. A total of 30 fractions were collected. Fractions 8-14 contained a substance with the same chromatographic mobility as 4 and were pooled. These fractions contained 84% of the total radioactivity eluted from the column.

Radiochemical purity was determined by co-chromatography of an aliquot of the combined fractions (10 μL) with a standard sample of cold 4 (4 μg) on TLC (20x20 cm, silica gel GHLF) using dichloromethane-acetone (5:1, v/v) as the solvent. The developed plates were visualized under short wavelength ultraviolet light and the position of 4 was marked. The plate was divided into 10 zones. The zones were scraped off separately with a razor blade into a counting vial and the radioactivity in each zone was measured. Radiochemical purity was calculated as the percent of total activity which co-chromatographed with the authentic sample of 4.

The combined fractions was concentrated under a slow stream of nitrogen. The residue was dissolved in ethyl acetate (2 mL) and transferred into a vial. The vial was placed in a lead-shielded container (3 mm thick) and stored at 0°C.



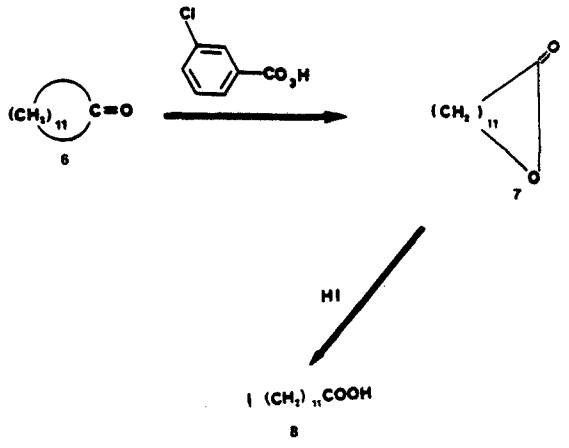
Scheme 1. Synthesis of 12-O-(12'-iodododecanoyl)phorbol-13-acetate.

RESULTS AND DISCUSSION

12-O-(12-Iodododecanoyl)phorbol-13-acetate (IPA, 4), an analog of TPA, was synthesized from phorbol (1) by a three step reaction sequence (Scheme 1). Phorbol (1) was obtained from croton oil. A number of diterpene alcohols, including phorbol, are present in croton oil primarily in the form of esters of fatty acids. Treatment of croton oil with barium hydroxide in methanol resulted in the hydrolysis of these esters and the liberation of the free alcohols (17,23). Phorbol was isolated and purified from the crude extract of the hydrolyzed oil by reversed-phase column chromatography (24). Selective acetylation of phorbol to provide phorbol-13,20-diacetate was accomplished by using acetic anhydride and triethylamine in a solvent mixture of dichloromethane-tetrahydrofuran (18).

12-Iodododecanoic acid (8) was obtained from cyclododecanone (6) by a two step reaction sequence (19) (Scheme 2). The ketone 6 was oxidized with MCPBA to the macrocyclic lactone dodecanolide (7). The lactone 7, without further purification, was cleaved with hydroiodic acid to provide the iodocarboxylic acid 8.

Phorbol-13,20-diacetate (2) was coupled with 12-iodododecanoic acid by the conventional DCC coupling procedure (20) to give 3. Selective cleavage of the C_{20} acetyl group in 3 with perchloric acid/methanol solution provided the



Scheme 2. Synthesis of 12-iodododecanoic acid.

desired product 4.

Isotope exchange by heating an acetone solution of 4 and sodium [^{125}I] iodide provided the desired ^{125}I-labeled product (5). This type of isotope exchange reaction yields products with moderate specific activities and relatively low incorporation of radioactivity (21,22). The product [^{125}I]-12-O-(12-iodododecanoyl)phorbol-13-acetate, was found to have 99% radiochemical purity after normal phase flash column chromatography. The specific activity of the product was 2.7 Ci/mmol. Approximately 8% of the radioactivity of sodium [^{125}I] iodide was incorporated in the product. [^{125}I]-12-O-(12-Iodododecanoyl)phorbol-13-acetate is currently used in examining the binding of phorbol esters to specific cell receptors.

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REFERENCES

1. Blumberg, P.M., C.R.C. Crit. Rev. Toxicol.; Vol. 7, 153, 199 (1980-1981).
2. Hecker, E., Fusenig, N.E., Kunz, W., Marks, F. and Thielman, H.W. (eds): Carcinogenesis--a comprehensive survey, vol. 7, Raven Press, New York, 1982.

3. Hecker, E. in Slaga, T.J., Sivak, A. and Boutwell, R.K. (eds): Mechanisms of tumor promotion and cocarcinogenesis, vol. 2, 1-588, Raven Press, New York, 1978.
4. Boutwell, R.K., C.R.C. Crit. Rev. Toxicol; Vol. 2, 419-443 (1974).
5. Sando, J.J. and Young, M.C., Proc. Natl. Acad. Sci, USA, 80, 2642, 1983.
6. Blumberg, P.M., Jaken, S., Konig, B., Sharkey, N.N., Leach, K.L., Jeng, A.Y. and Yeh, E., Biochemical pharmacology, 33, 933, 1984.
7. Dunn, J.A. and Blumberg, P.M., Cancer Research, 43, 4632, 1983.
8. Parker, P.J., Stabel, S. and Waterfield, M.D., EMBO Journal, 3, 953, 1984.
9. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y., J. Biol. Chem., 258, 11442, 1983.
10. Honikman-Leban, E. and Moule, Y., Gann, 75, 729, 1984.
11. Tran, P.L., Brienne, M.J., Malthete, J. and Lacombe, L., Tetrahedron Letters, 27, 2371, 1986.
12. Liskamp, R.M.J., Brothman, A.R., Arcoleo, J.P., Miller, O.J. and Weinstein, I.B., Biochem Biophys Res Comm, 131, 920, 1985.
13. Vandenbark, N.R. and Niedel, J.E., J Nat Cancer Inst, 73, 1013, 1984.
14. Gescher, A., Biochemical Pharmacology, 34, 2587, 1985.
15. Still, W.C., Kahn, M. and Mitra, A., J. Org. Chem, 43, 2923, 1978.
16. Rinehart, K.L., Jr., Science, 218, 254, 1982.
17. Crombie, L., Games, M.L. and Pointer, J.J., J. Chem. Soc., C., 1347, 1968.
18. Sorg, B., Furstenberger, G., Berry, D.L., Hecker, E. and Marks, F., J. Lipid Res. 23, 443, 1982.
19. Kruizinga, W.H. and Kellogg, R.M., J. Am. Chem. Soc., 103, 5183, 1981.
20. Marston, A. and Hecker, E., Z. Naturforschung, 38, 1015, 1983.
21. Counsell, R.E., Ranade, V.V., Blair, R.J., Beierwaltes, W.H. and Weinhold, P.A., Steroids, 16, 317 (1970).
22. Seevers, R.H. and Counsell, R.E., Chem. Rev., 82, 575, 1982.
23. Hecker, E. and Schmidt, R., Fortschr. Chem. Org. Naturst., 31, 377 (1974).
24. Mishra, N.C., Estensen, R.D. and Abdel-Monem, M.M., J. Chromatog., 369, 435 (1986).