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POSITRON LABELED PHORBOL ESTER: SYNTHESIS METHOD FOR "NON-CARRIER ADDED" PHORBOL 13-[1-^{11}C] BUTYRATE USING KETENE REACTION

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SUMMARY

A new method was developed to synthesize positron labeled phorbol esters for second messenger imaging based on a receptor binding to protein kinase C. [1-^{11}C] propyl ketene was produced by the pyrolytic decomposition of [1-^{11}C]butyric acid. This new method was made possible by the reaction of [1-^{11}C]propyl ketene to the hydroxyl group of phorbol under the non-carrier added conditions. The precursor protected in the 20-hydroxyl group of phorbol was acylated by [1-^{11}C]propyl ketene to the 13-hydroxyl group producing phorbol 13-[1-^{11}C]butyrate ([^{11}C]P13Bu). Total synthesis time was about 60 minutes and the specific activity was greater than 67 GBq/μmol (1800 mCi/μmol). [^{11}C] P13Bu will prove to be useful ligand for PET studies due to the simple procedures required for synthesis.

key words: Phorbol esters, Phorbol 13-butyrate, propyl ketene, protein kinase C, Second messenger imaging, PET

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INTRODUCTION

There is currently a great interest in protein kinase C[1] which is associated with neuronal functions. The role of protein kinase C in cell surface signal transduction through the phosphatidyl inositol system has been clarified[2)3)]. It has also been suggested that protein kinase C has a close connection with the central nervous system, that is, it has been found to be concentrated in the brain[4)5)]. As has already been established, phorbol 12,13-dibutyrate (PDBu) is suitable for the ligand to bind protein kinase C in in vitro studies[6)7)].

This study was conducted to derive a synthesis method of C-11 labeled phorbol esters, potentially useful positron emitting radioligands for PET studies. The labeling method described for the synthesis of phorbol 13-[1-^{11}C] butyrate ([^{11}C] P13Bu) depends on the acylation by a ketene compound[8)], which was produced by the pyrolytic decomposition of [1-^{11}C] butyric acid, of a hydroxyl group of phorbol under non-carrier added conditions. The new labeling technique by using [1-^{11}C] propyl ketene proved to be effective to obtain acylated compounds such as phorbol esters with a high specific activity.

MATERIALS AND METHODS

Phorbol 20-methoxytrityl ether (PMTE), phorbol 13-butyrate (P13Bu), phorbol 12-butyrate (P12Bu) and phorbol 12,13-dibutyrate (PDBu), were purchased from LC services Corporation (Woburn), and n-propyl bromide, lithium and 4-dimethylaminopyridine (DMAP) were purchased from Wako Pure Chemical Industries (Osaka). UV spectra were measured in hexane in 1 cm cells in a spectrophotometer (Shimadzu model SPD-6AV). Mass spectra were measured for P13Bu by SIMS (secondary ion MS; Hitachi M-80, acceleration voltage 9kV, xenon^{+}, matrix with m-nitrobenzyl alcohol) mass spectrometer and for propyl ketene by EIMS (electron impact MS; Hitachi M-80, direct inlet system) mass spectrometer. ^{1}H-NMR spectra were measured by NMR

spectrometer (JEOL GX-270).

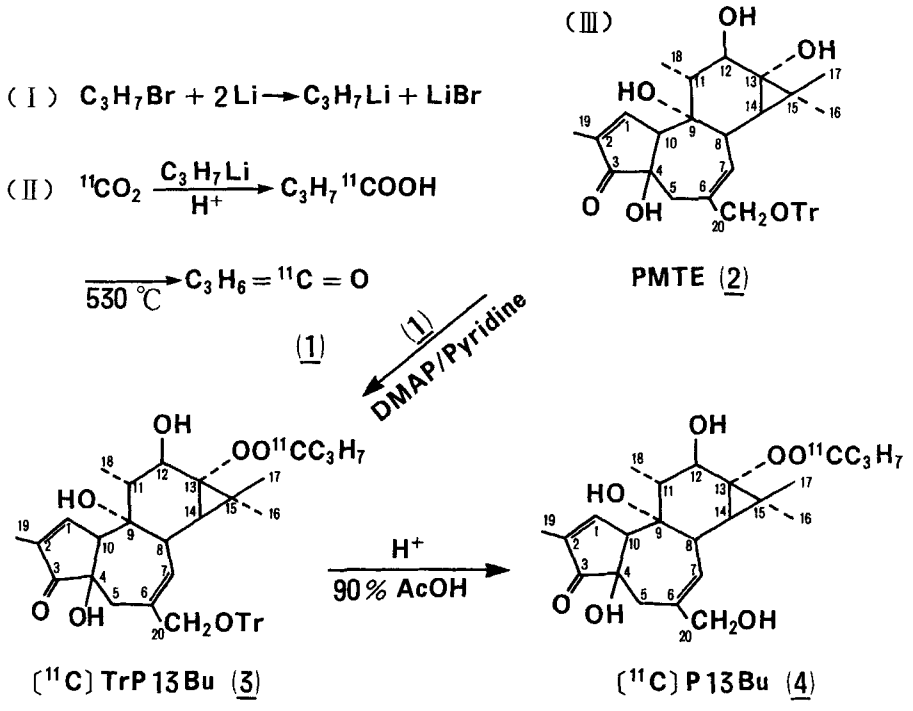
Synthesis of phorbol 13-butyrate employing propyl ketene reaction

Butyric acid vapor at room temperature in a carrier of helium gas (3.4 nmol/ml) was introduced (flow rate of 100 ml/min) to a quartz glass reaction tube containing quartz wool to facilitate thermal exchange at 530°C. The reactive product in the flow was bubbled into a glass reaction vessel containing a solution of phorbol 20-methoxytrityl ether (1 μ mol) and DMAP (1 μ mol) in 0.5ml of dry pyridine for 1 minute at room temperature. The residue was dissolved in 0.1 ml dichrolomethane after pyridine was evaporated under reduced pressure at room temperature, followed by a detritylation with 3 ml of 90% acetic acid for 15 minutes at 60°C. The reaction mixture was analyzed by HPLC (Zorbax SIL) with eluent of hexane / diethyl ether / isopropyl alcohol (10:2:1) at a flow rate of 1.8 ml/min. ^{1}H-NMR and mass spectra analyses were performed to identify with the standard material of P13Bu. The acylation to phorbol was carried out at several temperatures in order to determine the optimal conditions of the propyl ketene reaction.

Preparation of [1-^{11}C] Butyric acid ("Non-carrier added")

Propyl lithium was prepared by the reaction of propyl bromide (3.3 mmol) with lithium (14.4 mmol) in dry diethyl ether (30 ml) and the reaction mixture was stirred for 30 minutes at room temperature. The mixture was centrifuged after the termination of the reaction and the impurities were subsided. C-11 labeled carbon dioxide ($^{11}CO_2$) was produced by a $^{14}N(p,\alpha)^{11}C$ reaction using a cyclotron (JSW Baby Cyclotron system located at Nishijin Hospital) and $^{11}CO_2$ gas was trapped in a reaction vessel immersed in in liquid N_2. The supernatant (80 μ l) of propyl lithium solution was rapidly injected in a mixture of $^{11}CO_2$ and nitrogen carrier gas. A subsequent reaction of $^{11}CO_2$ with propyl lithum occurred after taking the reaction vessel out of the liquid N_2. Three minutes later 100μl of water was added to decompose the lithium complex. After the water in the mixture was evaporated under reduced pressure in a 60°C bath, the lithium salt of [1-^{11}C] butyric acid (1) was obtained.

Fig. 1. Reaction scheme used in the synthesis of [^{11}C]P13Bu.



Synthesis of phorbol 13-[1-^{11}C]butyrate

The scheme for the synthesis is shown in Fig. 1. 85% H_3PO_4 (50 μl) was added to the lithium salt. After the salt dissolves in the acid, 50 mg of P_2O_5 powder was added to the solution. The reaction vessel was connected to the quartz glass tube and the [1-^{11}C]butyric acid vapor was transferred by a helium gas flow from the heated glass vessel at 160°C to the heated quartz glass tube at 530°C. [1-^{11}C]propyl ketene on carrier helium gas (flow late 100 ml/min) was bubbled into a glass reaction vessel containing a solution of PMTE (2) (1 μmol) and DMAP (1 μmol) in 0.5 ml of dry pyridine. When the reaction was complete, pyridine was evaporated, followed by a detritylation as described above.

RESULT

All phorbol esters which were studied were analysed by HPLC under the same conditions as described in the caption below Tab. 1. There were two main ingredients in the end products, whose retention times were 3.2 and 7.3 minutes, respectively. The retention time of 7.3 minutes was identified as being that of standard P13Bu. The retention time of 3.2 minutes supposed to be that of tritylized P13Bu (TrP13Bu). The detritylized product was analized by ^{1}H-NMR and SIMS. The ^{1}H-NMR data gave good agreement with the value that had been obtained with the standard material of P13Bu. Identification data: ^{1}H-NMR ($CDCl_3$), δ(ppm) 1.3 (-$(CH)_2$-); 2.0 (-CH_2COO-); 2.5, 3.1, 4.0 and 5.6 (free hydroxyl groups). The mass spectra data by SIMS: m/e 417 $(MH-H_2O)^+$; 435 $(M+H)^+$; 457 $(M+Na)^+$; 869 $(2M+H)^+$.

Table 1. References of retention times of phorbol esters on HPLC analyses.

Phorbol esters	Retention times (minutes)
TrPDBu	1.8
TrP13Bu	3.2
PDBu*	4.3
P13Bu*	7.3
PMTE**	11.4
P12Bu*	12.9

HPLC conditions

Column: Zorbax SIL (Dupont Instrument), 4.6 mm x 25 cm

Mobile phase: Hexane / Ether / Isopropyl alcohol (10:2:1)

Flow rate: 1.8 ml/min; Column temperature: 25°C

Detector: UV wave length 240nm

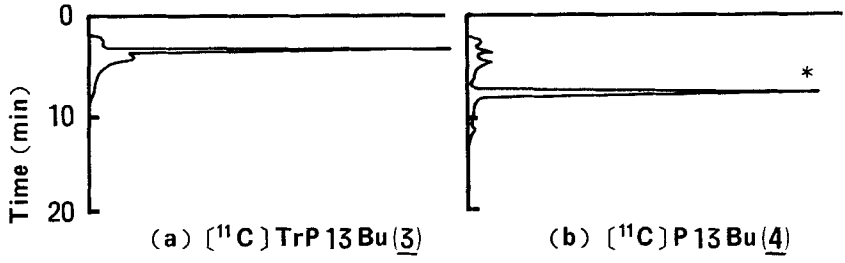
*Data taken from standard materials

**PMTE: Phorbol 20-methoxy trityl ether

Under the conditions of the cold run as described, the reaction within 1 minute produced one main product 20-methoxytrityl phorbol 13-butyrate (TrP13Bu). The acylation to the 13-hydroxyl group was shown to be very rapid. One minor product, 20-methoxytrityl phorbol 12,13-dibutyrate, accounted for less than 1% of the total products. However, the reaction over 3 minutes started to produce the diester and the amount of the minor product increased with time. Accordingly to begin with, the propyl ketene reaction produced a 13-monoester and the diester was produced in the next step.

Radiochemical yield of [1-^{11}C] butyric acid calculated based on trapped $^{11}CO_2$ was approximately 80%. Shown in Fig. 2 is the HPLC profile of the C-11 labeled propyl ketene adduct (3) of phorbol 20-methoxytrityl ether (2) in hot runs. The yield of acylation with [1-^{11}C] propyl ketene (1) to phorbol 20-methoxytrityl ether solution was approximately 16% of butyric acid. Total synthesis time was about 60min. Total radiochemical yield was 3.2% based on $^{11}CO_2$ at EOB. Radiochemical purity of final product ([^{11}C]-P13Bu (4)) fractionated by separative HPLC was greater than 98%. The specific activity of [^{11}C] P13Bu which was calculated from [^{11}C]TrP13Bu on radio HPLC was greater than 67 GBq/μmol (1800mCi/μmol) at EOS.

Fig. 2. Radio HPLC of [^{11}C]TrP13Bu and [^{11}C]P13Bu Conditions described in Tab. 1.

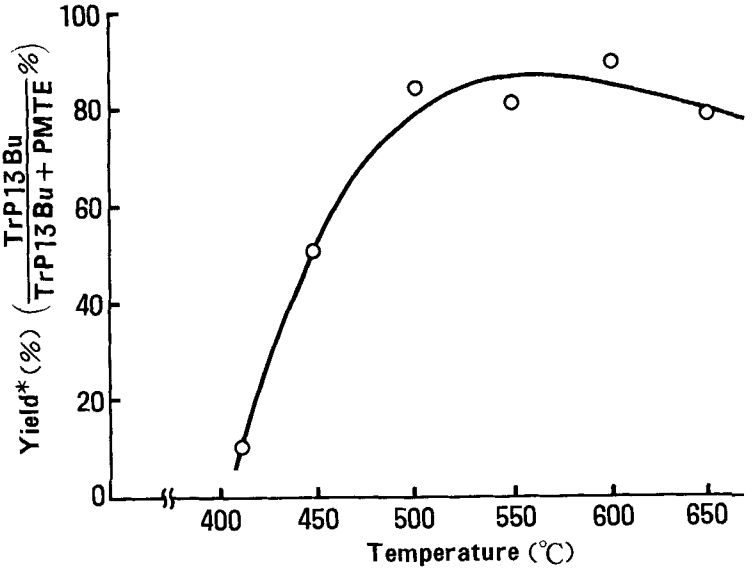


*** : The HPLC profile obtained before the process of separative HPLC was shown.**

DISCUSSION

This is the first report on the method of synthesis of C-11 labeled phorbol esters which should be useful positron emitting radioligands for PET studies. The labeling method depends on the use of [1-^{11}C]propyl ketene as an agent for labeling ligands such as phorbol esters. [1-^{11}C]propyl ketene was obtained by pyrolytic decomposition from the butyric acid. This method is appropriate for acylation of phorbol as it is an effective method with a high reactivity to the hydroxyl groups. In our preliminary study using R-^{11}COCl as an acylating agent, which was prepared from R-^{11}COOH with $SOCl_2$, the residual $SOCl_2$ could not be removed completely, producing ill effects on the protected groups of phorbol, especially in the 20-hydroxyl group. With a Ketene method, which was chosen to resolve this problem, it is easy to obtain a pure compound in an on line system. As shown in Fig. 3

Fig. 3. Optimal thermal conditions for propyl ketene production.



*: Yield is expressed as the ratio of TrP13Bu to the total of TrP13Bu and PMTE

the optimal temperature to produce propyl ketene was 530-550°C. This temperature must be strictly maintained, as propyl ketene will not be produced at temperatures under 450°C. It is clear that the reaction is dependent upon the temperature in the quartz reaction tube.

Generally ketene is an extremely unstable compound. However, the dimer formation of ketene will occur when ketene is concentrated at room temperature. Identification of the propyl ketene dimer was performed by EIMS (electron impact mass spectra). As a result there were several peaks suggested to be propyl ketene (m/e 71, $M+H^+$; 140, $2M^{+\cdot}$). Ion $M+H^+$ (m/e 71) is probably fragmental ion from the ketene dimer. Accordingly, it can be considered that PMTE was acylated by propyl ketene in this synthesis.

When the reaction was processed in a non-carrier added condition, the reactivity of the 13-hydroxyl group of PMTE was shown to be clearly higher than that of the 12-hydroxyl group. This tendency was shown even though in cold runs with another acylating agents when the agent was highly diluted. The only C-11 adduct that it is possible to obtain is [^{11}C]TrP13Bu. Conveniently enough, this selectivity facilitates the preparation of monoesters to be used as ligands for protein kinase C, that is, P13Bu has a higher affinity to the receptor than P12Bu, based on analogy to similar compounds[9]. Due to the high reactivity of the 20-hydroxyl group it is necessary to provide protection against the agent employed. The 20-hydroxyl group can resist acylation by ketene when using phorbol 20-methoxytrityl ether as a precursor. The 4- and 9- hydroxyl groups showed low reactivity and therefore ketene acylation did not occur even without protection.

The specific activity obtained by this method, determined by HPLC and UV detector, was shown to be very high and enough to perform ligand-receptor measurements in clinical applications. A 10 mCi injection of the tracer would contain 5.6 nmol of P13Bu. Assuming normal dilution in human blood, the concentration of the tracer would be less than 1.5 nMol, which is about one fifth of the Kd value for PDBu[10]. Further basic work is in progress to characterize P13Bu as a ligand for protein kinase C.

In this paper we mentioned only the preparation of [^{11}C]P13Bu. [^{11}C]-

P13Bu probably has measurable affinity as a ligand for one or more of the protein kinase C subtypes. Due to the simple procedures for synthesis of [^{11}C]P13Bu, it will be useful ligands for PET studies. A further process, in which the 12-hydroxyl group of [^{11}C]TrP13Bu was acylated with cold propyl ketene, produced phorbol 12,13-[1-^{11}C]dibutyrate which is the better characterized ligand for binding studies with protein kinase C (Imahori, Y. et al., manuscript in preparation). Many phorbol esters which have biological potencies have been reported[11)-13)]. The application of this method of synthesis enable the production of many other C-11 labeled phorbol esters.

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