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Preparation of Tritium-Labelled 12-O-Retinoylphorbol-13-acetate ([20-3H]-RPA)

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In contrast to established principles of radioactive labelling of diterpene esters such as TPA it was impossible to introduce tritium into the highly sensitive 12-O-retinoylphorbol-13-acetate (RPA) via reduction of its cold 20-aldehyde with [3H]-sodium borohydride. As an alternative successful procedure, [20-3H]-phorbol-13-acetate was prepared at first. It was protected in position 20 by reaction with tritylchloride and the tritylether reacted with retinoic acid. In both reactions appropriate phorbol acetates were used as easy to separate cold carriers. After removal of the 20-tritylether group, [20-3H]-RPA of a specific activity was obtained that was sufficient for the compound to be used as a tool in biochemical investigations.

Introduction

12-O-Retinoylphorbol-13-acetate (RPA), the retinoyl analogue of the standard tigliane type tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), is highly skin irritant in mouse skin [2, 3]. Quantitatively, its irritancy is comparable with that of TPA. However, in the initiation/promotion protocol on the back skin of NMRI mice RPA is a very weak promoter, in contrast to TPA. This difference in biological properties is reminiscent of that reported for the highly unsaturated TPA-analogue Euphorbia factor Ti8 and TPA (see chart) and other unsaturated, aliphatic diterpene esters (DTE). This particular structure/activity relation supports the generalization previously deduced that irritancy is a necessary but insufficient requirement for tumor promotion in skin [4]. Moreover, it was shown that Euphorbia factor Ti8, RPA and, to a certain extent, also milliamine C may be used operationally in the initiation/promotion protocol on skin of NMRI mice as “incomplete” promoters in contrast to “complete” promoters, such as TPA and other DTEs of the daphnane (e.g. simplexin) and ingenane (e.g. 3-TI) type [5, 6]. Such subdivision of the stage of promotion in skin of NMRI mice in two substages, PI and PII (see Fig.), suggests the existence of a qualitative difference in the mechanism of action of “complete”

Abbreviations: DTE (diterpene ester); hplc (high performance liquid chromatography); LSC (Liquid Scintillation Counter); RPA (12-O-retinoylphorbol-13-acetate); rtlc (radio-tlc); tlc (thin-layer chromatography); TPA (12-O-tetradecanoylphorbol-13-acetate); [20-3H]-RPA (tritium-labelled 12-O-retinoylphorbol-13-acetate).

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Chart. Irritant and promoting activities of TPA and of the analogous, highly unsaturated DTE Ti8 and RPA indicate that in mouse skin irritancy is a necessary, yet incomplete requirement for promotion.
(e.g. TPA) and “incomplete” promoters (e.g. RPA). Such difference was detected recently when a qualitative difference in chromosomal damage was shown for TPA and RPA [7, 8, 9].

To allow for a better understanding of biochemical events critical for tumor promotion, studies on the distribution and metabolism of RPA in comparison to TPA appear most desirable (see l.c. [10]). Therefore, a procedure was developed to prepare tritium-labelled RPA, following the general outline of that previously devised for tritium-labelled TPA [11] and 3-TI [12], taking special account of the extreme sensitivity of RPA, even if unlabelled (see l.c. [3]).

**Experimental**

**Materials and methods**

For TLC precoated silica gel plates were used (60 F254; Merck, Darmstadt, FRG): in analytical tlc plates of 10×20 cm, layer thickness/0.25 mm; in preparative tlc plates of 20×20 cm, layer thickness 0.50 mm. The regions of interest were detected by cochromatography of non-radioactive reference compounds which were detected at 254 nm. To isolate compounds the silica gel was extracted with ethyl acetate. RTLC was carried out with a linear analyzer (Berthold, Wildbad, FRG).

HPLC was performed using a reversed phase column (Ultrasound ODS; 4.6×250 mm; 5 μm; Beckman) or a normal phase column (Zorbax sil 4.6×250 mm; 5 μm; Du Pont). To monitor the radioactivity profiles, the eluate was subfractioned and the radioactivity measured in a LSC.

**Products.** Labelling with [3H]-sodiumborohydride (82 Ci/m mole) was done according to instructions (see l.c. [1], with minor modifications) by custom synthesis (Amersham-Buchler, Braunschweig, FRG).

Dry pyridine was purchased from Merck (Darmstadt, FRG) and dry N,N-dimethylformamide from Aldrich (Steinheim a.A., FRG). Unlabelled phorbol derivatives were synthesized according to published procedures [13]. All other substances used were as pure as available.

**Reactions and general work-up procedure.** Reactions and handling involving retinoic acid and derivations were done in yellow light. The reaction mixtures were extracted with ethyl acetate. The organic extract was subsequently washed with ice-cold 1M—HCl solution, 0.5 M phosphate buffer (pH 7) and brine (each time 3×2 ml) and dried with anhydrous sodium sulfate. The yield indicates the portion of desired product as % of the total radioactivity.

**Phorbol-13-acetate-20-aldehyde (2)**

1 was oxidized to the corresponding 20-aldehyde with manganese dioxide in dichloromethane [11]. The product was purified by tlc in dichloromethane/ethyl acetate 1/3 and subsequently crystallized from dichloromethane/cyclohexane. Yield: >80% of 2.

**[20-3H]-Phorbol-13-acetate (3)**

7 mg (17.2 nmole) of 2 was reduced with 50 mCi (specific activity: 82 Ci/m mole) of sodium-boro-[3H]-hydride in 750 μl of ethanol [11]. 3 was purified by tlc in ethyl acetate/water (saturated with ethyl acetate) = 7/3. Yield: 48% of 3; nominal specific activity: 20.5 Ci/m mole.

**[20-3H]-Phorbol-13-acetate-20-tritylether (4)**

To a solution of 24 mCi 3 in ethyl acetate 14 mg (31 μmole) of phorbol-12,13-diacetate was added as a carrier. The solvent was evaporated and the dry residue was dissolved in 100 μl of dry pyridine. 50 mg (180 μmole) of tritylchloride was dissolved in
400 μl of dichloromethane and added to the pyridine solution. The flask was flushed with argon. After three days of stirring at room temperature the reaction was stopped by adding 1 ml of brine. The mixture was worked up as described above. For purification of the desired product (including removal of the carrier derived phorbol-12,13-diacetate-20-trityl-ether) preparative tlc was used (four plates; ethyl acetate/petroleum ether = 1/1). Yield: 80% of 4.

[20-3H]-12-O-Retinoylphorbol-13-acetate-20-trityl-ether (5)

To a solution of 19 mCi of 4 11.2 mg (25 μmole) of phorbol-13,20-diacetate in ethyl acetate was added as a carrier. The solvent was removed under reduced pressure. The dry residue was dissolved in 90 μl of dichloromethane containing 0.8 mg (6.6 μmole) of 4-(N,N-dimethylamino)pyridine. In a separate flask, 36.8 mg (123 μmole) of retinoic acid was dissolved in 100 μl of dry N,N-dimethylformamide and 400 μl of dichloromethane. The solution was chilled to 0 °C; subsequently 27.6 mg (134 μmole) of N,N'-dicyclohexylcarbodiimide was added. After 10 minutes the second solution was transferred to the reaction flask. The flask was flushed with argon. After one day stirring at room temperature one ml of brine was added. Work-up procedure as described above. For removal of dicyclohexylurea the residue was suspended in 10 ml of heptane/ethyl acetate = 4/1 and filtered through a 0.45 μm filter (Millex HV). The filter was rinsed with ice-cold solvent twice. The crude products were purified by tlc (first: four plates; second: two plates; solvent system: ether/petroleum ether = 1/1) twice. Yield: 41% of 5. Unchanged 4 was reisolated in a yield of 43%.

[20-3H]-12-O-Retinoylphorbol-13-acetate (6)

7.5 mCi of 5 was dissolved in 50 ml of methanol plus 100 μl of 70% HClO4. After 15 minutes under argon the reaction was stopped by adding about 100 mg of sodium acetate. Solvent was removed and the residue suspended in 5 ml of water. The suspension was worked up as described above (except the acid wash). Crude 6 was purified by tlc in ethyl acetate/petroleum ether = 2/1 and subsequently by reversed phase hplc (methanol/water = 92/8; 1.0 ml/min). Yield: 47.5% of 6 (± 3.6 mCi).

The identity of 6 was proven by hplc using a normal phase column [hexane/ethyl acetate (0.05% of water)/2-propanol = 300/150/2; 1.5 ml/min] and a reversed phase column (methanol/water = 92/8; 1.0 ml/min). The eluate was fractionated and radioactivity was monitored in a LSC.

The specific activity of 6 was determined by comparing the extinction at 366 nm in methanol/water = 92/8 of the labelled compound with a calibration curve made using unlabelled RPA. Radioactivity was determined in a LSC. Specific activity: 18.5 Ci/mmmole.

Storage

6 was stored in ethyl acetate at −20 °C under argon [3]. The flask was wrapped in a black foil.

Results and Discussion

In the standard procedure of phorbolester labeling, the ester was oxidized to its 20-aldehyde by manganese dioxide, and afterwards reduced by [3H]-sodiumborohydride [11]. However, the conjugated double bonds present in RPA suggested not to apply this method. Therefore, in analogy to the synthesis of cold RPA [2], phorbol-13-acetate was labelled in the usual manner [11], protected in position 20 as tritylether, esterified with retinoic acid in position 12, and deprotected to yield [20-3H]-RPA.

By the conventional methods [13], neither the alcohol group in 20-position could be protected selectively by tritylation or acetylation, nor could the hydroxyl group in 12-position be acylated by retinoic acid successfully in [20-3H]-phorbol-13-acetate. Despite a large excess of the reactants mentioned above over [20-3H]-phorbol-13-acetate, none of the desired products was detectable even after several days. On the other hand, experiments in which the concentration of phorbolester was increased by addition of appropriate cold phorbolestes indicated that the concentration of phorbol ester may be essential. Since a high specific activity of [20-3H]-RPA is required for its use in biochemical investigations, the concentration of phorbolester cannot be increased using cold phorbol-13-acetate, because it would lower the specific activity of the product. Therefore, two other phorbol derivatives appropriate for the specific stage of the reaction sequence were used as carriers. The carriers and their products could be separated easily from the labelled product desired, using the differences in their chromatographic properties.

Following these principles, [20-3H]-phorbol-13-acetate was tritylated in 20-position using phorbol-12,13-diacetate as carrier. The resulting labelled tritylether was successfully acylated with retinoic acid using the carbodiimid method by addition of phorbol-13,20-diacetate as carrier. Finally, the cleav-
age of the trityl ether by methanol/HClO₄ yielded the desired [20-³H]-RPA. The identity was confirmed by hplc on normal and reversed phase columns. As a rule, the specific activity of the [20-³H]-RPA obtained is as high as that of the starting material, [20-³H]-phorbol-13-acetate. The yields of [20-³H]-RPA and the intermediate products were comparable to those obtained in the synthesis of unlabelled compounds [2].

Thus 4 was shown to be an important intermediate which may be used for the synthesis of other interesting phorbol derivatives not accessible by standard methods, e.g. Euphorbia factor T₁₈.

With [20-³H]-RPA as a tool the distribution and metabolism in vivo [10] and in vitro [14] was investigated. The affinity of [20-³H]-RPA to the phorbol ester receptor was also determined [15].