On the Chemistry of Ingenol, V*
Preparation of Tritium-Labeled 3-O-Tetradecanoylingenol ([20-3H]-3-TI)
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Ingenol Ester, Tumor Promoter, Tritium-Labeling

Tritium-labeling of the ingenol monoester 3-O-tetradecanoylingenol (3-TI), the prototype
tumor promoter of the ingenane type diterpene esters, is described. The preparation starts with
oxidation of 3-TI by manganese dioxide to yield 3-TI-20-aldehyde which is reduced by [3H]-
sodium borohydride in the presence of equimolar amounts of cerium trichloride hexahydrate
to give [20-3H]-3-TI. The labeled promoter is obtained in 42% overall yield with a specific ra-
dioactivity of 15.8 Ci/mmol.

Introduction
A wide range of skin irritant ingenol-3-esters has been isolated from many species of the botanical
family of Euphorbiaceae especially from the genus Euphorbia or were prepared by partial syntheses
from ingenol [1–4]. Many of them turned out to be tumor promoters in the two-stage model of tu-
morogenesis on mouse skin [5–7]. In addition, in current efforts to develop certain Euphorbia
species as renewable oleochemical resources, 3-O-hexadecanoylingenol (3-HI) may be considered a
potential risk factor of occupational cancer [8,9].

Also, 3-HI elicits and modulates a variety of bio-

logical and biochemical responses in various types
of cultured cells, including release of prostaglandin
E2 in mouse peritoneal macrophages [10], induc-
tion of Epstein-Barr virus (EBV) associated pro-
tins in EBV-transformed lymphoblastoid cell
lines derived from Burkitt’s Lymphoma [11], stim-
ulation of choline incorporation in the human ep-
thelial cell line HeLa [12], suppression of sponta-
neous and prevention of lymphokine-induced en-
hancement of rat macrophage cytolytic activity
[13], and induction of differentiation in the human
promyelocytic leukemia cell line HL-60 [14]. Spe-
cific high-affinity receptors for biologically active
ingenol-3-esters have been found in different types
of cultured cells [15], and in subcellular fractions
from mouse epidermis [16] and mouse brain [17,18].

Recent studies of structure/activity relations in-
vestigating the effect of chain-length on the irritant
and tumor promoting activities of aliphatic ingen-
ol-3-esters have demonstrated a biphasic re-
sponse with a maximum at a chain-length of 14
carbon atoms [7, 19, 20]. These results led to the
suggestion of 3-O-tetradecanoylingenol (3-TI, for
structure compare chart 1) as the most appropriate
standard for biological and biochemical investiga-
tions on the mechanism of tumor promotion by in-
genane type diterpene esters [7].

With the exception of the tigliane type diterpene
ester 12-O-tetradecanoylphorbol-13-acetate (TPA)
and some other phorbol esters [2, 21–26], very lit-
tle is known about the metabolic fate of tumor
promoters of mouse skin. The information availa-
ble indicates that metabolic conversion is not re-
quired for biological activities but, in fact, leads to
a loss of activities [2, 25, 26]. To clarify whether
this is a general rule applicable to other types of
diterpene ester tumor promoters, it is timely to
investigate the metabolism of the ingenane type
tumor promoter 3-TI. In connection with this and
other studies requiring radioactive 3-TI, a proce-
dure for the preparation of tritium-labeled 3-TI
was developed which is reported in this article.

Abbreviations: EBV, Epstein-Barr virus; 3-HI, 3-O-
hexadecanoylingenol; HPLC, high performance liquid
cromatography; HPTLC, high performance thin layer
cromatography; TLC, thin layer chromatography; Rr
retention time; TPA, 12-O-tetradecanoylphorbol-
13-acetate; 3-TI, 3-O-tetradecanoylingenol.
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Experimental

Materials and methods

[1]H]-Sodium borohydride (specific radioactivity 64 Ci/mmol) was purchased from NEN, Dreieich, FRG. Sodium borodeuteride was from Aldrich, Steinheim, FRG, and cerium trichloride hexahydrate was from Fluka, Buchs, Switzerland. Rotiszint 44 was purchased from Roth, Karlsruhe, FRG. Ingenol was prepared from the seed oil of Euphorbia lathyris [1].

To monitor the reactions, silica gel 60 F$_{254}$ pre-coated TLC aluminium sheets from Merck, Darmstadt, FRG, were used. Spots were visualized under UV light (254 nm) and by spraying with vanillin/sulfuric acid followed by brief heating at 100 °C. R$_f$-values were determined on silica gel and RP-18 F$_{254}$ pre-coated HPTLC plates from Merck, Darmstadt, FRG.

HPLC separations in analytical scale were done on a chromatograph from Waters, Eschborn, FRG, which consisted of two series 6000 A pumps, a model 660 solvent programmer, a model U 6 K injector, and a series 440 absorbance detector which was operated at 280 nm. Normal-phase separations were carried out on a 250 × 4.6 mm I.D. Zorbax Sil column from DuPont, Bad Nauheim, FRG, preceeded by a 50 × 4.6 mm I.D. guard column packed with “Zorbax uncoated” donated by DuPont, Bad Nauheim, FRG. Reversed-phase separations were performed on a 250 × 4.6 mm I.D. Ultraspere ODS column from Beckman, München, FRG, preceeded by a 50 × 4.6 mm I.D. guard column packed with the same material. The flow rate was 1.5 ml/min.

For preparative HPLC, a 830 Preparative High-Performance Liquid Chromatograph with UV detector (254 nm) from DuPont, Bad Nauheim, FRG, was used. Purifications were carried out on a 250 × 21.2 mm I.D. Zorbax Sil straight-phase column and a 250 × 21.2 mm I.D. Zorbax C-8 reversed-phase column both from DuPont, Bad Nauheim, FRG.

The radiochemical purity of [20-3H]-3-TI was determined by reversed-phase HPLC using acetonitrile/methanol/water = 69/23/8 as eluent. Fractions of the eluent (1.5 ml) were collected into scintillation vials and mixed with 10 ml Rotiszint 44. Radioactivity was determined in a Betaszint BF 8000 scintillation counter from Berthold, Wildbad, FRG.

Mass spectra were run on a M 711 mass spectrometer from Varian, Bremen, FRG. 1H NMR spectra (90 MHz) were recorded with a HX-90 from Bruker Physics, Karlsruhe, FRG, using tetramethylsilane (δ = 0.00) as internal standard and CDCl$_3$ as solvent.

Phosphate buffer (pH about 7) was prepared from 80 g sodium dihydrogen phosphate monohydrate and 20 g disodium hydrogen phosphate dihydrate in 11 water.

3-O-Tetradecanoyligenol-20-aldehyde

Manganese dioxide (4.2 g) was added to a stirred solution of 3-TI (169 mg, 0.3 mmol) in dichloromethane (17 ml). After 10 min, the manganese dioxide was removed by decantation and filtration (Millex-SR disposable filter), and the filtrate evaporated to dryness in vacuo. The resulting yellow resin was subjected to preparative HPLC on the silica gel column using dichloromethane/ethyl acetate/petroleum ether = 10/60/30, with chamber saturation). 0.3 mL was collected and partitioned between brine (150 ml) and cyclohexane (2 × 300 ml). The organic extracts were combined, dried (magnesium sulfate), and evaporated to dryness in vacuo. Yield: 71%. R$_f$ = 0.22 (silica gel HPTLC plates, ethyl acetate/petroleum ether = 1/1, with chamber saturation), 0.26 (RP-18 HPTLC plates, water/acetonitrile/ethyl acetate = 10/60/30, without chamber saturation); R$_f$ = 18.0 min (RP-18 HPLC, methanol/water = 92/8). 1H NMR: δ = 1.08, 1.10 (6H, s, s; H$_3$-1, H$_3$-7); 1.27 (s; alkyl-CH$_3$), 1.78 (3H, t, H$_3$-19); 1.26 (2H, m; -O-CO-CH$_3$-); 2.38 (2H, m; -O-CO-CH$_3$-); 3.57 (1H, s, exchangeable with D$_2$O; OH-4), 3.91-4.26 (5H, m, 1H exchangeable with D$_2$O; H-5, H-8, H$_3$-20, OH), 5.46 (1H, s; H-3), and 5.94-6.12 (2H, m; H-1, H-7). MS: m/z = 558 (M$^+$).

3-O-Tetradecanoyligenolen-20-3H-acetate

The radiochemical purity of [20-3H]-3-TI was determined by reversed-phase HPLC using acetonitrile/methanol/water = 69/23/8 as eluent. Fractions of the eluent (1.5 ml) were collected into scintillation vials and mixed with 10 ml Rotiszint 44. Radioactivity was determined in a Betaszint BF 8000 scintillation counter from Berthold, Wildbad, FRG.

Mass spectra were run on a M 711 mass spectrometer from Varian, Bremen, FRG. 1H NMR spectra (90 MHz) were recorded with a HX-90 from Bruker Physics, Karlsruhe, FRG, using tetramethylsilane (δ = 0.00) as internal standard and CDCl$_3$ as solvent.

Phosphate buffer (pH about 7) was prepared from 80 g sodium dihydrogen phosphate monohydrate and 20 g disodium hydrogen phosphate dihydrate in 11 water.

3-O-Tetradecanoyligenol (3-TI)

3-TI was prepared by acylation of ingenol-5,20-acetonide with 4-(N,N-dimethylanilino)pyridine/tetradecanoyl chloride in toluene, followed by treatment of the resulting 3-TI-5,20-acetonide with perchloric acid in methanol, analogous to the preparation of 3-O-decanoyligenol described previously [19]. After work-up of the mixture a colorless resin was obtained which was purified by preparative HPLC on the reversed-phase column using methanol/water = 92/8 as eluent. The 3-TI peak was collected and partitioned between brine (150 ml) and cyclohexane (2 × 300 ml). The organic extracts were combined, dried (magnesium sulfate), and evaporated to dryness in vacuo. Yield: 71%. R$_f$ = 0.22 (silica gel HPTLC plates, ethyl acetate/petroleum ether = 1/1, with chamber saturation), 0.26 (RP-18 HPTLC plates, water/acetonitrile/ethyl acetate = 10/60/30, without chamber saturation); R$_f$ = 18.0 min (RP-18 HPLC, methanol/water = 92/8). 1H NMR: δ = 1.08, 1.10 (6H, s, s; H$_3$-1, H$_3$-7); 1.27 (s; alkyl-CH$_3$), 1.78 (3H, t, H$_3$-19); 2.38 (2H, m; -O-CO-CH$_3$-); 3.57 (1H, s, exchangeable with D$_2$O; OH-4), 3.91-4.26 (5H, m, 1H exchangeable with D$_2$O; H-5, H-8, H$_3$-20, OH), 5.46 (1H, s; H-3), and 5.94-6.12 (2H, m; H-1, H-7). MS: m/z = 558 (M$^+$).

3-O-Tetradecanoyligenolen-20-aldehyde

Manganese dioxide (4.2 g) was added to a stirred solution of 3-TI (169 mg, 0.3 mmol) in dichloromethane (17 ml). After 10 min, the manganese dioxide was removed by decantation and filtration (Millex-SR disposable filter), and the filtrate evaporated to dryness in vacuo. The resulting slightly yellow resin was subjected to preparative HPLC on the silica gel column using dichloromethane/ethyl acetate/petroleum ether = 97/3/0.1 as eluent. Collection of the 3-TI-20-aldehyde peak followed by evaporation gave rise to 94 mg (56% yield) of a clear resin. R$_f$ = 0.56 (silica gel HPTLC plates, ethyl acetate/petroleum ether = 1/1, with chamber saturation), R$_f$ = 21.0 min (RP-18 HPLC, methanol/water = 92/8). 1H NMR: δ = 1.09 (6H, s, s; H$_3$-16, H$_3$-17); 1.26 (s; alkyl-CH$_3$), 1.77 (3H, m; H$_3$-19), 2.37 (2H, m; -O-CO-CH$_3$), 3.53 (1H, s, exchangeable with D$_2$O; OH-4), 4.29 (1H, m, forms
singlet on D$_2$O exchange; H-5), 4.61 (1 H, m; H-8), 4.76 (1 H, d, exchangeable with D$_2$O; OH-5), 5.72 (1 H, s; H-3), 5.99 (1 H, m; H-1), 7.10 (1 H, m; H-7), and 9.31 (1 H, s; H-20). MS: m/z = 556 (M$^+$.)

Reduction of 3-O-tetradecanoylingenol-20-aldehyde furnishing 3-O-tetradecanoylingenol

3-TI-20-aldehyde (22.2 mg, 0.04 mmol) was dissolved in a solution of cerium trichloride hexahydrate (14.2 mg, 0.04 mmol) in ethanol (0.5 ml). After cooling to 0 °C, a solution of sodium borohydride (0.75 mg, 0.02 mmol) in ethanol (0.5 ml) was added with stirring. After 5 min, the solution was partitioned between phosphate buffer (10 ml) and cyclohexane (2×5 ml). The organic extracts were combined, dried (magnesium sulfate), and evaporated to dryness in vacuo. HPLC separation of crude 3-TI was carried out as described above. Yield: 80%. $^1$H NMR and mass spectra were identical to those of authentic 3-TI.

[20-$^2$H]-3-O-tetradecanoylingenol

[20-$^2$H]-3-TI was prepared from 3-TI-20-aldehyde by reduction with sodium borodeuteride analogous to the procedure described for 3-TI (see above). $^1$H NMR: $\delta = 1.08, 1.10$ (6 H, s, s; H$_3$-16, H$_3$-17), 1.27 (s; alkyl-CH$_2$), 1.78 (3 H, m; H$_3$-19), 2.38 (2 H, m; –O–CO–CH$_2$–), 3.57 (1H, s, exchangeable with D$_2$O; OH-4), 3.91–4.26 (4 H, m, 1H exchangeable with D$_2$O; H-5, H-6, H-20, OH), 5.46 (1H, s; H-3), and 5.94–6.12 (2 H, m; H-1, H-7). MS: m/z = 559 (M$^+$).

[20-$^3$H]-3-O-tetradecanoylingenol

3-TI-20-aldehyde (8.3 mg, 15 $\mu$mol) was dissolved in a solution of cerium trichloride hexahydrate (5.3 mg, 15 $\mu$mol) in ethanol (0.68 ml). After cooling to 0 °C, a solution of [3H]-sodium borohydride (2.5 $\mu$mol, 160 mCi) in ethanol (0.32 ml) was slowly (1–2 min) added with stirring. The mixture was allowed to react for 5 min, and, after addition of phosphate buffer (10 ml), was extracted with cyclohexane (2×5 ml). The organic extracts were combined, dried (magnesium sulfate), and evaporated to dryness in vacuo. The crude product was purified by two HPLC runs, first on the analytical straight-phase column using hexane/ethyl acetate = 1/1 as eluent, followed by the analytical reversed-phase column using acetonitrile/methanol/water = 69/23/8 as eluent. Radiochemical purity of [20-$^3$H]-3-TI was >99%. Specific radioactivity of [20-$^3$H]-3-TI as determined by reversed-phase HPLC peak area integration and scintillation counting was 15.8 Ci/mmol. Identification of [20-$^3$H]-3-TI was effected by cochromatography with non-radioactive 3-TI as reference compound in the HPLC systems described above.

Storage

[20-$^3$H]-3-TI was stored in ethyl acetate (1 mCi/ml) at −70 °C without any detectable decomposition taking place within two years.

Results and Discussion

The strategy for tritium-labeling of the ingenane type diterpene ester 3-TI was to selectively oxidize the primary hydroxyl group in position 20 to an aldehyde group which, in a second step, could be selectively reduced by treatment with [3H]-sodium borohydride to give [20-$^3$H]-3-TI (compare Scheme 1). In the case of tigliane type diterpene esters including TPA and other phorbol esters a similar procedure led to the desired products tritium-labeled in the 20 position of the diterpene moiety [24, 27].

The starting material 3-TI-20-aldehyde was obtained from 3-TI by oxidation with manganese dioxide in dichloromethane essentially as described for the preparation of TPA-20-aldehyde.

![Scheme 1](image)

Scheme 1. The tumor promotor 3-O-tetradecanoylingenol (3-TI) and the reaction pathway used for its labeling with tritium.
from TPA [28–30]. However, attempts to obtain 3-TI from 3-TI-20-aldehyde by reduction with sodium borohydride analogous to the procedure described for the preparation of TPA from TPA-20-aldehyde [27] were unsuccessful, since under these conditions the conjugated carbon-carbon double bond in the 6,7 position of 3-TI was reduced in addition to the aldehyde group in position 20 yielding as the predominant product 6,7-dihydro-3-TI rather than 3-TI [31].

Previous studies investigating sodium borohydride reduction of conjugated aldehydes and ketones have shown that in general 1,4 reduction is a competing process leading to substantial amounts of the fully saturated alcohols [32, 33], while application of sodium borohydride in conjunction with lanthanoide chlorides strongly favours 1,2 reduction yielding the allylic alcohols as the predominant or exclusive products [33, 34]. Of lanthanoide tested, cerium has been shown to give the highest regioselectivity with most enones [35] and was therefore selected as the most appropriate catalyst for sodium borohydride reduction of 3-TI-20-aldehyde.

Application of this method to the reduction of 3-TI-20-aldehyde by treating an equivalent amount of 3-TI-20-aldehyde and cerium trichloride hexahydrate in ethanol with sodium borohydride at 0 °C completely prevented the formation of 6,7-dihydro-3-TI and afforded the desired product 3-TI exclusively and in good yield. This method was adapted for the synthesis of tritium-labeled 3-TI. To ensure quantitative utilization of the [3H]sodium borohydride, the starting material 3-TI-20-aldehyde was present in excess of this reagent. HPLC separation of the reaction mixture on silica gel efficiently removed the unreacted starting material, but produced a by-product corresponding to unlabeled 5-O-tetradecanoylingenol. This by-product was completely removed by reversed-phase HPLC to give better than 99% radiochemically pure tritium-labeled 3-TI which displayed chromatographic properties identical to those of authentic unlabeled 3-TI. The position of the label was established indirectly by applying sodium borodeuteride as a reducing agent. 1H NMR and mass spectroscopic data of the product indicated that one deuterium atom had been specifically introduced at position 20. Specific radioactivity of the final product [20-3H]-3-TI as determined by reversed phase HPLC peak area integration and scintillation counting was 15.8 Ci/mmol, and the yield in relation to the added [3H]-sodium borohydride was 42%.

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