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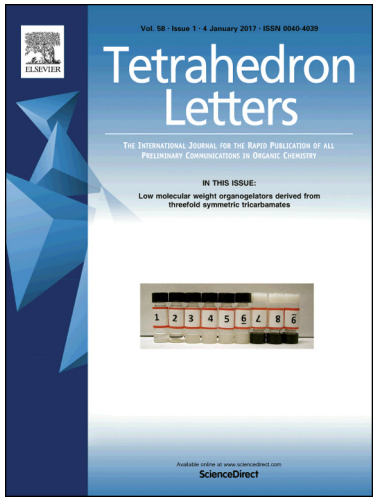

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A convenient method for the preparation of 20-[^{18}O]-labelled ingenol

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Abstract: A short and efficient protecting group-free synthesis of isotopically labeled 20-[^{18}O]-ingenol has been developed. Based on a highly selective (only one out of four hydroxy groups) Mitsunobu reaction of ingenol with $^{18}O_2$-acetic acid and subsequent methanolysis, this route yielded the desired 20-[^{18}O]-ingenol in high yield and chemical and isotopic purity.

Keywords: ingenol; labeled compounds; natural products; protecting group-free synthesis; mass spectroscopy.

Nature is a virtually endless source of new biologically active compounds, novel chemical skeletons, and thus represents an infinite pool of novel drug candidates. Although many isolated compounds have proven to be very interesting from a biological activity view point,[1] in many cases the limited quantity obtainable from the original source has hampered their further development as drug candidates. In some cases, it was found that alternative natural sources, e.g. different plants or fungi, also contained the targeted compounds, or derivatives exploitable on an industrial scale (e.g. taxol).[2] In our laboratory, we have been involved in the development of a rapid, efficient and precise method for metabolome-containing quantification – known as the isotope dilution method.[3] In recent years, this method has been extensively used in plant metabolomics to determine the quantities of compounds (metabolites) of interest in the plant metabolome. The advantage of this method is that it permits excellent control over the loss and/or degradation of analytes (compounds of interest) during the extraction and purification processes. One hurdle, however, is the necessity to produce isotopically labeled standards of these compounds.

Recently, we have been interested in the detection and determination of plants containing high amounts of ingenol (**1**), a diterpenoid compound first isolated in 1968 from the seed oil of the leafy shrub *Croton tiglium* (Euphorbiaceae).[4] Ingenol itself can be found in plants as a conjugate with various carboxylic acids in the form of esters or polyesters. Many of these esters possess interesting anti-tumor and/or anti-HIV activities.[5] One such compound, ingenol 3-angelate (**2**), an ester conjugate of ingenol and angelic acid, was recently approved for the topical treatment of actinic keratosis – a precancerous skin condition.

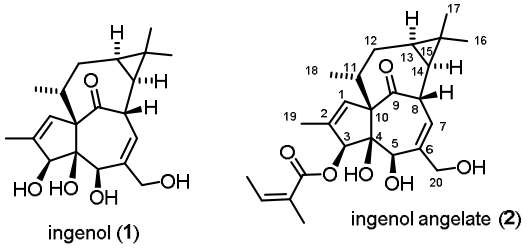


Figure 1. Structure of ingenol (**1**) and ingenol angelate (**2**)

Despite the tremendous efforts that the synthetic community have devoted over the last few decades to the total synthesis of ingenol (**1**), the *de novo* synthesis of **1** remains elusive. Notably, Baran and co-workers[6] recently published a fairly short and efficient route for the synthesis of **1** in 14 steps. Ingenol-3-angelate **2** has been prepared by semisynthesis in 3 steps from ingenol **1** isolated from the seeds of carper spurge (*Euphorbia lathyris*; yield ~100 mg.kg^{-1}).[7] In natural sources, ingenol **1** is present as various mono-, di- and tri-ester conjugates. Consequently, free ingenol **1** is available only upon the methanolysis of these extracts.[8]

As noted above, we wished to find plants containing the highest amounts of ingenol **1** and then use the isotope dilution method to carry out the quantification. To do so, we required an isotopically labeled standard that would have a molecular peak of [M+2] compared to naturally occurring ingenol **1**.[9] Previously, the derivative of ingenol **1** fulfilling this criterion bearing a ^{3}H atom at the C-20 position was prepared[10] (Figure 2). Because we expected to work with rather substantial quantities of the labeled compound, it was decided to avoid the use of the ^{3}H-labelled compound (^{3}H-**1**). As such, a di-deuterated derivative d_2-**1** was envisioned to be used instead, thus, requiring the incorporation of two deuterium atoms to ingenol **1** for the [M+2] molecular ion to be obtained. Based on the MS-MS spectra analysis of ingenol, we speculated that the introduction of isotopic labels to the C-20 position would be the simplest and most straightforward approach.[12]

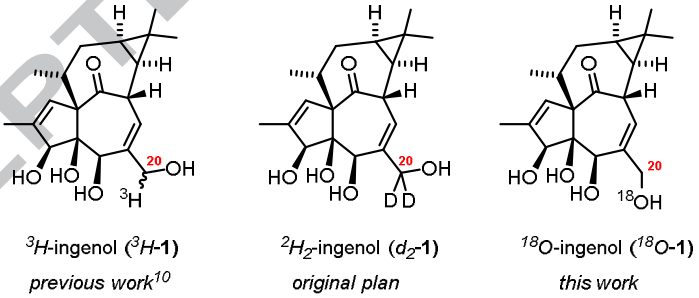
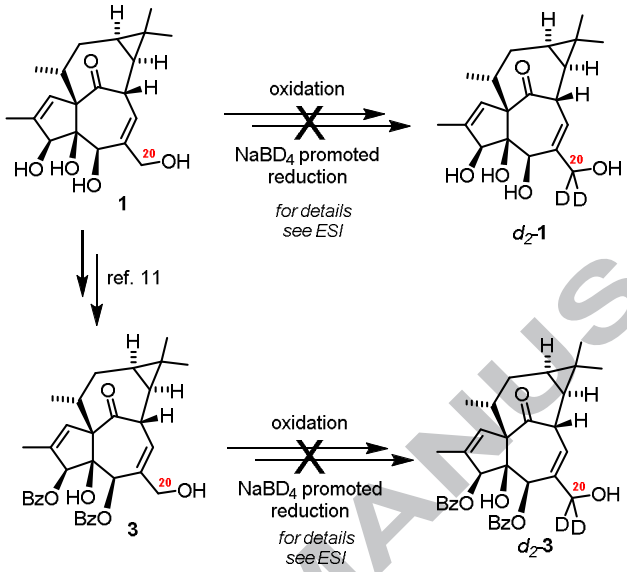


Figure 2. Isotopicaly labelled ingenol derivative exploitable by the isotopic dillution method.

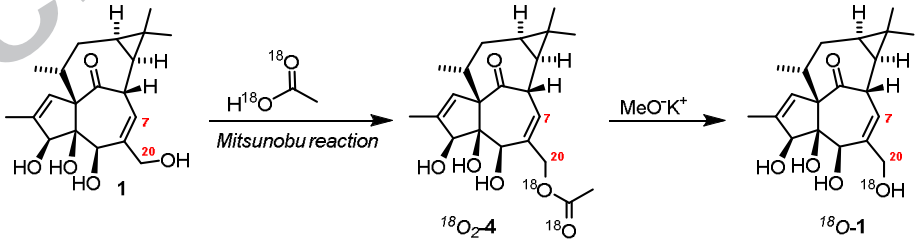
Initially, we envisioned the preparation of $^{2}H_2$-ingenol (d_2-**1**) *via* a protecting group-free synthesis based on an iterative sequence of selective C-20 allylic alcohol oxidation and $NaBD_4$ reduction (Scheme 1). Unfortunately, the low yielding C-20 alcohol oxidation step and non-selective $NaBD_4$-promoted reduction (several products formed) resulted in the critical failure of this approach.[9] Similar results were obtained when the bis-benzoylated ingenol derivative **3**[11] was used as the starting material in the oxidation/reduction sequence.[9]

With these results in hand, we therefore reconsidered our approach to the required ingenol derivative. Based on MS/MS spectra analysis of ingenol **1**,[12] it was decided to prepare C-20 ^{18}O labeled ingenol (^{18}O-**1**, Scheme 2). We assumed that the primary allylic alcohol at C-20 could be selectively reacted with $^{18}O_2$-acetic acid under Mitsunobu reaction conditions in the presence of the three remaining hydroxy groups.[9] Methanolysis of the resulting $^{18}O_2$-acetylated ingenol $^{18}O_2$-**4** would then accomplish the synthesis of ^{18}O-**1**.



Scheme 1.

Model Mitsunobu and methanolysis reactions were first attempted with glacial acetic acid (Table 1). It was observed, that in all cases the substitution reaction proceeded selectively at the C-20 hydroxy group, with no product arising from possible S_N2' substitution of C-7 detected. Upon further experimentation, a significant solvent-independent reactivity difference between the DEAD and DIAD reagents was observed. When DEAD was used as an activating agent (Entries 1–5), the conversion of ingenol **1** did not proceed to completion (generally 70-85% conversion). When additional DEAD reagent was added (Entry 4), several side-products were observed in the crude reaction mixture, however, complete consumption of **1** was still not achieved.

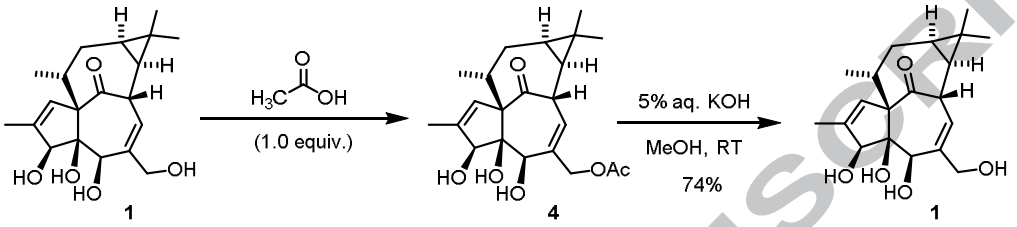


Scheme 2.

The use of DIAD assured the full conversion of **1** to **4** in THF or in benzene, and subsequent methanolysis of acetate **4** yielded ingenol **1** in good overall yields.

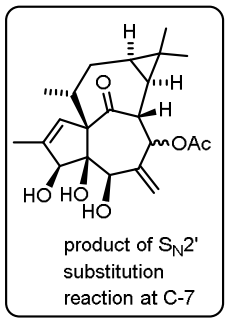
The optimized reaction conditions were then applied to the synthesis of ^{18}O-**1**[13] (Scheme 3), and the desired product was obtained in two steps from ingenol **1** in 63% yield and excellent chemical (>97%) and isotopic (95% ^{18}O-incorporation) purity.[9] Finally, the MS/MS spectra of the prepared ^{18}O-**1** ingenol derivative confirmed our expectations that the major MS-fragment [$M_{ingenol}$-OH]$^+$ included the ^{18}O-hydroxy group and that it can be used as a standard for ingenol **1** quantification *via* the isotope dilution method.[9]

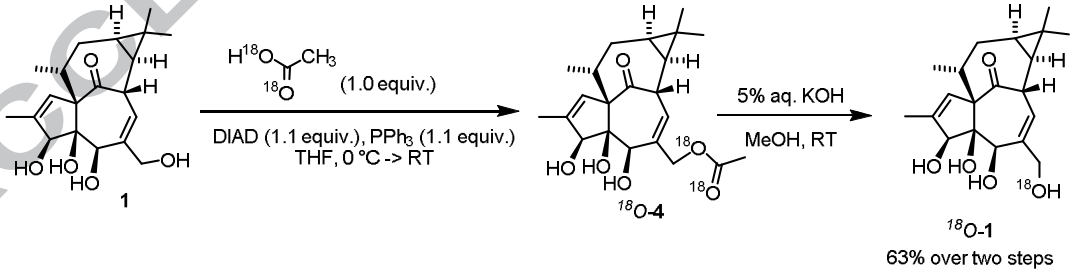
Table 1. Model Mitsunobu and methanolysis reactions



Entry	Conditions	Conversion of **1** [%]	Isolated yield of **4** [%]
1	DEAD (1.1 equiv.), PPh_3 (1.1 equiv.) THF, 0 °C -> RT	85%	68%
2	DEAD (1.1 equiv.), PPh_3 (1.1 equiv.) THF, RT	75%	49%
3	DEAD (1.1 equiv.), PPh_3 (1.1 equiv.) benzene, RT	75%	38%
4[a]	DEAD (1.1 equiv.), PPh_3 (1.1 equiv.) THF, 0 °C -> RT	85%	52%
5	DEAD (1.1 equiv.), PBu_3 (1.1 equiv.) THF, 0 °C -> RT	77%	61%
6	DIAD (1.1 equiv.), PPh_3 (1.1 equiv.) THF, 0 °C -> RT	>98%	91%
7	DIAD (1.1 equiv.), PBu_3 (1.1 equiv.) THF, 0 °C -> RT	>98%	72%
8	DIAD (1.1 equiv.), PPh_3 (1.1 equiv.) benzene, RT	>98%	58%

[a] additional DEAD (0.2 equiv.) and PPh_3 (0.25 equiv.) added after 1 h





Scheme 3.

In conclusion, we have developed a short and efficient protecting group free synthesis of ^{18}O-labelled ingenol ^{18}O-**1**. The targeted compound was prepared in excellent overall yield and chemical and isotopic purity. The location of ^{18}O-introduction was carefully chosen, so the ^{18}O-oxygen atom

remains within the molecule of ingenol **1** during the MS analysis.[9] The prepared labeled compound has successfully been used to determine the ingenol content in various plant extracts.[14]

Acknowledgements

The financial support by the Ministry of Education, Youth and Sports of the Czech Republic (grant LO1204 from the National Program of Sustainability I) is gratefully acknowledged. The Teva Czech Industries company is gratefully acknowledged for the generous financial and material support and generous gift of ingenol. We are grateful to Dr. Miloslav Chudík and Dr. Ladislav Cvak (TEVA Czech Industries) for inspiring discussions and valuable advice.

Supplementary Material

Characterization data for all new compounds and experimental procedures. This material is available free of charge via the internet.

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12. For detailed MS/MS analysis of ingenol **1** fragmentation, see ESI.
13. Synthesis of 20-^{18}O-ingenol (*^{18}O*-**1**): A solution of ingenol **1** (300 mg, 0.86 mmol, 1.0 equiv.), $^{18}O_2$-acetic acid (52 µL, 0.9 mmol, 1.05 equiv.) and PPh_3 (263 mg, 0.9 mmol, 1.05 equiv.) in dry THF (9 mL, 0.1 M) was cooled to 0 °C and the resulting mixture stirred for 5 min prior to DIAD (142 µL, 0.9 mmol, 1.05 equiv.) addition. The resulting mixture was stirred at 0 °C for an additional 2 h and then at RT for 12 h. The resulting mixture was evaporated to dryness under reduced pressure and the residue purified by column chromatography on silica gel ($CHCl_3$/EtOAc = 4:1->2:1->1:0->0:100) to give 314 mg (92%) of 20-$^{18}O_2$-acetate ingenol ($^{18}O_2$-**4**). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.10 (dd, *J* = 4.7, 1.4 Hz, 1H), 5.94 (q, *J* = 1.5 Hz, 1H), 4.71 (d, *J* = 12.5 Hz, 1H), 4.52 (d, *J* = 12.7 Hz, 1H), 4.44 (s, 1H), 4.09 (broad dd, *J* = 11.3, 3.5 Hz, 1H), 3.68 (s, 1H), 2.38 – 2.30 (m, 1H), 2.27 (ddd, *J* = 15.6, 8.9, 3.1 Hz, 1H), 2.06 (s, *J* = 3.4 Hz, 3H), 1.85 (d, *J* = 1.4 Hz, 3H), 1.80 – 1.72 (m, 1H), 1.27 (dt, *J* = 9.9, 7.2 Hz, 1H), 1.11 (s, 3H), 1.07 (s, 3H), 0.97 (d, *J* = 7.0 Hz, 3H), 0.71 (td, *J* = 8.6, 6.3 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 207.1, 171.4, 139.1, 136.9, 132.3, 128.7, 84.5, 80.7, 73.9, 72.8, 66.9, 44.2, 40.0, 31.2, 28.7, 24.1, 23.3, 23.1, 21.4, 21.3, 17.6, 15.7. $^{18}O_2$-

acetylated ingenol (*[18]O*-**4)** (100 mg, 0.25 mmol, 1.0 equiv.) was dissolved in MeOH (5 mL) and the resulting mixture stirred at 0 °C for 5 min. A 5% aqueous KOH solution (15 mL) was added to the reaction mixture over a 5 min. period and the resulting mixture then allowed to warm to RT. After 30 min at RT the mixture was extracted with EtOAc (3 x 25mL) and combined organic layers washed with brine (10 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel ($CHCl_3$/EtOAc = 2:1->1:1->0:100) yielding (50.8 mg, 57%) of ^{18}O-ingenol (*[18]O*-**1)**. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.05 (dd, *J* = 5.1, 1.0 Hz, 1H), 5.94 (q, *J* = 1.5 Hz, 1H), 4.40 (s, 1H), 4.17 (d, *J* = 12.7 Hz, 1H), 4.12 (d, *J* = 12.7 Hz, 1H, partial overlap), 4.11 (ddd, *J* = 10.4, 6.5, 2.4 Hz, 1H), 3.81 (s, 1H), 2.35 – 2.25 (m, 2H), 1.85 (d, *J* = 1.2 Hz, 3H), 1.79 – 1.71 (m, 2H), 1.71 – 1.39 (broad s, 2H), 1.20 (t, *J* = 7.0 Hz, 1H), 1.11 (s, 3H), 1.05 (s, 3H), 0.96 (d, *J* = 7.0 Hz, 3H), 0.93 (dd, *J* = 11.8, 8.4 Hz, 1H), 0.69 (td, *J* = 8.6, 6.3 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 208.5, 140.8, 139.4, 129.0, 127.4, 84.3, 80.00, 75.0, 72.8, 60.6, 44.2, 39.3, 31.2, 28.7, 24.0, 23.5, 23.0, 17.7, 15.7, 14.3.

14. Results of ingenol and its conjugates analysis in some species of the *Euphorbia genus* by ultra-high performance liquid chromatography-tandem mass spectrometry using the isotope dilution method were presented at the 63rd International Congress and Annual Meeting of the Society-for-Medicinal-Plant-and-Natural-Product-Research, Budapest (Hungary), Meeting abstract PW = 180. Abstract is included in Beres, T.; Dragull, K.; Pospisil, J.; Tarkowska, D.; Dancak, M.; Dolezal, K.; Strnad, M. *Planta Medica* **2015**, *81*, 1541.

Highlights

- Short and efficient 2 step synthesis of labeled ingenol.
- Protecting group free introduction of the ^{18}O atom with excellent selectivity.
- Highly selective Mitsunobu reaction.
- $^{18}O_2$-acetic acid as the ^{18}O atom source.

Graphical Abstract

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