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Research Article

Synthesis of all-*trans*-[10′-^{3}H]-8′-apo-β-carotenoic acid

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Summary

The enzyme, 15,15′-β-carotene dioxygenase (BCDOX), facilitates the oxidation of β-carotene to yield retinal. This is a remarkable process in which one of 11 double bonds in β-carotene is selectively oxidized. To further probe the mechanistic aspects of BCDOX, the synthesis of all-*trans*-[10′-^{3}H]-8′-apo-β-carotenoic acid is reported. This compound will be used as a photoaffinity labeling reagent to probe the β-carotene binding pocket within BCDOX. The synthesis outlines a simple and efficient route for the incorporation of tritium at the 10′ olefinic carbon of 8′-apo-β-carotenoic acid. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: 15,15′-β-carotene dioxygenase; 8′-apo-β-carotenoic acid; photoaffinity labeling

Introduction

The need for carotenoids in the human diet has been well established. Either the carotenoids or their oxidation products, particularly retinoid compounds, are essential in various pathways such as the visual transduction, gene regulatory control, cancer, and other disease prevention.[1–7] Although animals cannot produce retinoids such as vitamin A, they can convert β-carotene (**1**) (a tetraterpenoid

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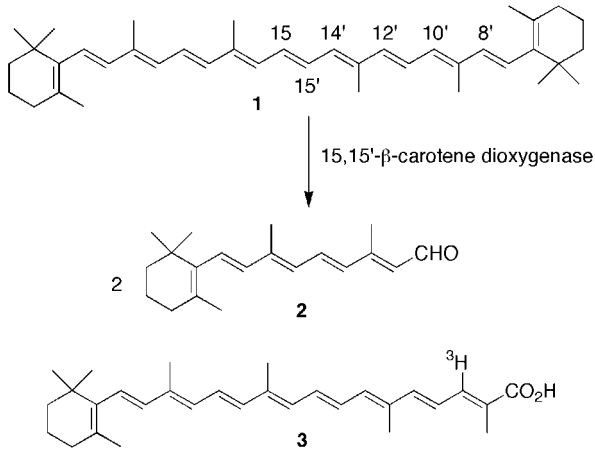


Scheme 1.

hydrocarbon with 11 double bonds) via a dioxygenase, which with remarkable selectivity cleaves the central olefin to yield retinal (**2**) (Scheme 1).[8–10] This enzyme, 15,15'-β-carotene dioxygenase (BCDOX), was first isolated by Goodman and Huang, but has not received a great deal of attention until very recently.[11] The past few years has witnessed a resurgence of activity in this area, mainly as a result of the identification and isolation of the gene responsible for BCDOX in a number of organisms.[12–17] In view of the growing interest in the physiological implication of retinoids in human health,[1,2,7,18–20] and the intricate interplay of the concentration of different retinoids and their physiological role,[1,18,21–24] it seems prudent to understand fully the nature, function, regulation, and mechanism of BCDOX.

Oxidative cleavage of β-carotene is an unprecedented process for a dioxygenase enzyme. Typically, dioxygenases require a hydrophilic handle such as a hydroxyl group to proceed, such as the case with intra- and extra-diol catechol dioxygenases.[25] Also, the catalytic mechanism for the enzymatic cleavage of a somewhat featureless hydrocarbon such as β-carotene (**1**) with such high regiospecificity is not well understood. Enzyme-substrate interactions that lead to stereospecific catalytic action through hydrogen bonding and dipolar communication are common. Understanding the enzymatic action on hydrocarbon substrates can shed light onto nature's methodology for utilizing other molecular interactions for recognition and specificity. Our interest in the mechanistic aspects of BCDOX has led us to synthesize the tritiated

photoaffinity label [10′-^{3}H]-8′-apo-β-carotenoic acid (**3**) to probe the binding pocket of β-carotene within BCDOX.

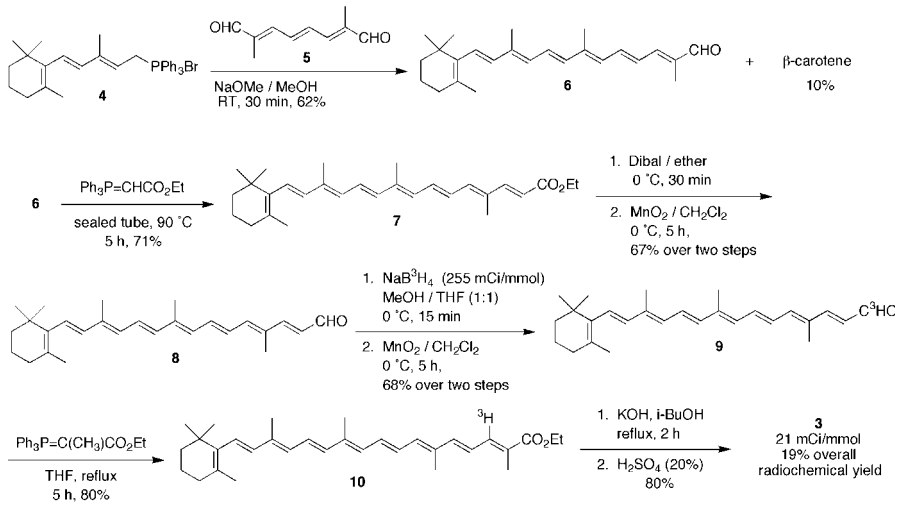
The choice of **3** as the photoaffinity probe stems from the fact that apo-β-carotenoic acids are substrates for BCDOX, and thus will bind the enzyme.[26–28] On the other hand, $\alpha,\beta-$unsaturated polyene carboxylic acids are capable of photoactivation and can be used as photoaffinity probes. This was demonstrated by Rando and coworkers during the isolation of retinoic acid binding proteins in which radiolabeled retinoic acid was used as the photoaffinity labeling agent.[29] In a similar manner, our first attempt at photoaffinity labeling of BCDOX will entail the use of **3**, the synthesis of which is reported here.

Results and discussion

Synthesis of radiolabeled carotenoids are challenging for two reasons. First, the polyolefinic nature of these compounds leads to their less than desirable stability, and thus introduction of radiolabels is generally limited to mild reactions. Although there is not a large body of literature on the synthesis of radiolabeled carotenoids (mostly biosynthesized by feeding of labeled precursors to various organisms),[30,31] related compounds such as retinoids have been synthesized by reduction of intermediate carbonyl groups with Na/LiB^3H_4, or semihydrogenation of intermediate acetylenes with $^3H_2$.[32–36] Secondly, their sensitivity to light forces the need to work in darkrooms with minimal red lighting and increases the technical difficulty of routine chemical manipulations.

Prudent with any radiolabeling, it is preferable to incorporate the label as close to the final step as possible. This will reduce the need for handling, and purification of radiolabeled intermediates, and increase the radioactive yield of the labeled compound. Although there are a range of positions present in 8′-apo-β-carotenoic acid (**3**) that could be considered as appropriate to incorporate tritium, we opted to place it on the 10′ carbon. We chose to use of NaB^3H_4 as the source of tritium, and thus required a reductive step to incorporate the label. C10′ was chosen as a suitable site after several trial non-radioactive syntheses of **3** were performed, in which the last reductive step was utilized for tritiation, and the subsequent steps could be performed in high yields, without the need for extensive manipulation and purification.

The synthesis of **3**, depicted in Scheme 2, followed the $C_{15}+C_{10}$ approach used by a number of researchers for the synthesis of



Scheme 2.

analogous structures.[37] Construction of the C_{15}-ylide **4** and 2,7-dimethyl-2, 4, 6-octatrienedial (**5**) was uneventful and followed reported procedures.[38,39] Wittig olefination of **5** with ylide **4** was initiated with NaOMe in methanol and delivered the C_{25}-aldehyde **6** in good yields with 97% *E* stereoselectivity of the newly formed double bond. Double olefination of **5** led to the formation of *β*-carotene (**1**) in 10% yield, however, it was easily removed owing to its hydrophobic nature. Treatment of aldehyde **6** with (carbethoxymethylene)triphenylphosphorane in a sealed tube at 95°C for 5 h led to **7** (>97% *E*) in 71% yield. Ethyl ester **7** was reduced with DIBAL, and the resulting allylic alcohol was oxidized with MnO_2 to provide C_{27}-aldehyde **8**. In our hands, commercially available oxidant did not yield any product, and led to the decomposition of starting material. However, freshly prepared activated MnO_2 worked well.[40] Also, maintainance of the temperature at 0°C was critical for the success of this oxidation.

The tritium label was incorporated by reduction of aldehyde **8** with NaB^3H_4 at 0°C, which was immediately reoxidized with MnO_2 to yield the radiolabeled C_{27}-aldehyde **9**. Although past reductions of *α,β*-unsaturated carbonyls have sometimes given rise to other reductive products such as 1,4-reduced compounds, none were observed in this instance. Yields reported in Scheme 2 beyond the radioactive reduction with NaB^3H_4 refer to isolated and purified products from the non-radioactive reactions, which were used to optimize each step towards

the synthesis of **3**. During the radioactive synthesis, crude radioactive intermediates were carried forward without isolation to minimize the number of manipulations and avoid loss of radioactive compound. The final compound was purified and fully characterized. Wittig olefination of **9** with (carbethoxyethylidene)triphenyl-phosphorane in refluxing THF proceeded well to deliver ester **10** as the sole product (*Z* isomer was not detected by NMR spectroscopy). The resulting triphenylphosphine oxide and remaining phosphonium salts were easily removed by flushing the crude reaction mixture through a short pad of silica. Hydrolysis of ethyl ester **10** proved to be more challenging than expected and routine basic and acidic conditions did not lead to a high yielding process. However, procedures detailed by a recent report by Larsen *et al.*[41] for hydrolysis of such esters was used successfully (see experimental) to obtain the radiolabeled acid **3** (21 mCi/mmol) in 19% overall radiochemical yield. With **3** in hand, we are presently in the process of optimizing photoaffinity labeling conditions. In conclusion, the present procedure provides a simple and efficient route for the synthesis of all-*trans*-[10′-^{3}H]-8′-apo-*β*-carotenoic acid (**3**).

Experimental

All reactions were carried under an atmosphere of nitrogen and removal of solvents was performed under reduced pressure with a Buchi rotary evaporator. THF and Et_2O were freshly distilled from sodium/benzophenone, and CH_2Cl_2 was distilled over CaH_2 under a nitrogen atmosphere. Radioactive NaB^3H_4 was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Analytical TLC was carried out using Merck 250 μm Silica gel 60 F_{254} and spots were visualized under UV light. Column chromatography was conducted using Silicycle silica gel (230–400 mesh). 300 MHz ^{1}H-NMR and 75 MHz ^{13}C-NMR spectra were recorded on a Varian Gemini-300 instrument, and the residual protic solvent ($CDCl_3$ or DMSO-d_6) was used as internal reference. UV-visible spectra were recorded on a Perkin-Elmer Lambda 40 spectrometer. A Wallac WinSpectral 1414 liquid scintillation counter was used for quantification of radioactivity. In a typical measurement 1–10 μl of sample was added to 3 ml of Optiphase 'HiSafe' 3 liquid scintillation cocktail (Wallac) and the solution was counted for 1 min. All the reactions were carried out in a darkroom under minimal photographic safety red lights.

*2,7,11-Trimethyl-13-(2,6,6-trimethyl-cyclohex-1-enyl)-trideca-2,4,6,8,10,12-hexaenal (**6**)*

Sodium methoxide (121.0 mg, 2.250 mmol, 1.5 eq.) was added to a solution of **4**[39] (817.0 mg, 1.5 mmol) in dry methanol (8 ml) at 0°C, and stirred until the phosphonium salt was completely converted to the deep red phosphorane. A solution of 7-dimethyl-2,4,6-trienedial **5**[38] (164.0 mg, 1 mmol) in dry methanol (**1** ml) was added and the mixture was stirred for 30 min. The reaction mixture was diluted with water (10 ml) and extracted with diethyl ether (3×5 ml). The organic layer was dried over Na_2SO_4 and the solvent was removed in *vacuo* to give the C_{25}-aldehyde **6** (217.0 mg, 62%, 97:3 *E:Z*), and β-carotene **1** (57.0 mg, 10%), which was purified by flash silica chromatography (2% ethyl acetate in hexane).

UV: λ_{max} (hexanes) 414 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.40 (1H, s), 7.03 (1H, d, J= 12 Hz), 6.96 (1H, dd, J = 14.4, 12.3 Hz), 6.77 (1H, dd, J = 14.4, 11.4 Hz), 6.41 (1H, dd, J = 14.4, 11.7 Hz), 6.5 (1H, d, J= 11.7 Hz), 6.7 (1H, d, J= 12.3 Hz), 6.12 (3H, m), 2.00 (3H, s, CH_3), 1.97 (3H, s, CH_3), 1.86 (3H, s, CH_3), 1.67 (3H, s, CH_3), 1.97–1.40 (6H, m, $3 \times CH_2$), 1.00 (6H, s, $2 \times CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.38, 148.94, 141.72, 137.88, 137.71, 137.42, 136.66, 136.22, 130.73, 130.30, 129.74, 127.74, 127.63, 127.16, 39.51, 34.17, 33.03, 28.89, 26.81, 21.70, 19.13, 12.97, 12.77, 9.51.

4,9,13-Trimethyl-15-(2,6,6-trimethyl-cyclohex-1-enyl)-pentadeca-2,4,6,8,10,12,14-heptaenoic acid ethyl ester (7)

A solution of **6** (110.0 mg, 0.310 mmol) and (carbethoxymethylene)-triphenylphosphorane (323.0 mg, 0.930 mmol) in dry THF (4 ml) was heated at 95°C for 5 h in a sealed tube. The reaction mixture was cooled to room temperature and diluted with water (10 ml) and Et_2O (10 ml). Phases were separated and the aqueous phase was extracted with Et_2O (2×10 ml). The combined organics were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Crude product was purified by flash silica chromatography (5% ethyl acetate in hexane) to yield **7** (93.0 mg, 71%, >97% *E*). The coupling constant for the newly formed double bond confirms the *E* stereochemistry. The *Z* isomer was not detected by NMR spectroscopy.

UV: λ_{max} (hexanes) 423 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 (1H, d, J= 15.6 Hz), 6.78 (1H, d, J= 12.9 Hz), 6.79 (1H, dd, J = 14.7, 11.4 Hz), 6.65 (1H, dd, J = 13.5, 11.7 Hz), 6.45 (1H, d, J= 11.1 Hz),

6.33 (1H, d, J= 14.7 Hz), 6.23 (1H, d, J= 11.7 Hz), 5.96 (3H, m), 5.84 (1H, d, J= 15.6 Hz), 4.20 (2H, q, J= 7.2 Hz), 2.01, (2H, m, CH_2), 1.97 (3H, s, CH_3), 1.96 (3H, s, CH_3), 1.89 (3H, s, CH_3), 1.69 (3H, s, CH_3), 1.60 (2H, m, CH_2), 1.45 (2H, m, CH_2), 1.28 (3H, t, J= 7.2 Hz, OCH_2 $\underline{CH_3}$), 1.00 (6H, s, $2 \times CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.51, 148.72, 139.25, 138.93, 137.79, 137.58, 136.91, 136.73, 133.74, 133.36, 131.57, 130.56, 129.54, 128.60, 127.16, 126.28, 116.23, 60.17, 39.55, 34.20, 33.07, 28.92, 21.75, 19.18, 14.31, 12.90, 12.76, 12.53.

*4,9,13-Trimethyl-15-(2,6,6-trimethyl-cyclohex-1-enyl)-pentadeca-2,4,6,8,10,12,14-heptaenal (**8**)*

To a cold (0°C) solution of **7** (90.0 mg, 0.215 mmol) in dry Et_2O (6 ml) was added 1 M solution of DIBAL in cyclohexane (0.43 ml, 0.430 mmol) and stirred for 30 min. Saturated sodium–potassium tartrate solution (3 ml) and glycerol (5 drops) were added. The reaction mixture was stirred over night at room temperature; phases were separated and the aqueous phase was extracted with Et_2O (3×10 ml). The combined organic layers were washed with brine (3 mL), dried over Na_2SO_4, filtered and concentrated in *vacuo* to give the corresponding allylic alcohol, which was pure enough to be used without further purification (80.0 mg, 99%).

UV: λ_{max} (hexanes) 400, 422 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.67 (3H, m), 6.22 (7H, m), 5.84 (1H, dt, J = 12, 6 Hz), 4.22 (2H, t, J = 6.0 Hz), 2.00 (2H, m, CH_2), 1.94 (6H, s, $2 \times CH_3$), 1.89 (3H, s, CH_3), 1.70 (3H, s, CH_3), 1.60 (2H, m, CH_2), 1.45 (2H, m, CH_2), 1.00 (6H, s, $2 \times CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 137.82, 137.68, 137.06, 136.68, 136.27, 136.09, 134.62, 132.32, 131.96, 130.68, 130.35, 129.35, 127.28, 126.66, 125.14, 63.91, 39.55, 34.21, 33.05, 28.92, 21.73, 19.20, 12.82, 12.78, 12.71.

MnO_2[40] (100.0 mg, 1.160 mmol, 20 eq.) was added to a stirred solution of the aforementioned allylic alcohol (22.0 mg, 0.058 mmol) in CH_2Cl_2 (3 ml) at 0°C and stirred for 5 h. The reaction mixture was filtered through a pad of celite and washed with CH_2Cl_2 (4×0.5 ml). Solvent was removed under reduced pressure and the crude aldehyde was purified by flash silica column chromatography (5% ethyl acetate in hexane) to yield **8** (15.0 mg, 68%).

UV: λ_{max} (hexanes) 436 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.57 (1H, d, J= 7.8 Hz), 7.13 (1H, d, J= 15.3 Hz), 6.82 (1H, dd, J = 13.8, 11.7 Hz), 6.73 (1H, dd, J = 14.7, 11.4 Hz), 6.53 (2H, m), 6.35 (1H, d,

$J = 15$ Hz), 6.25 (1H, d, $J = 12$ Hz), 6.14 (4H, m), 2.01(2H, m, CH_2), 2.00 (3H, s, CH_3), 1.96 (3H, s, CH_3), 1.94 (3H, s, CH_3), 1.70 (3H, s, CH_3), 1.60 (2H, m, CH_2), 1.45 (2H, m, CH_2), 1.01(6H, s, $2 \times CH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.74, 156.62, 141.21, 140.06, 137.81, 137.56, 137.44, 136.57, 135.36, 133.61, 131.43, 130.50, 129.73, 128.36, 127.53, 127.08, 126.93, 39.61, 34.25, 33.11, 28.95, 21.76, 19.21, 12.99, 12.82, 12.70.

*NaB^3H_4 reduction of **8** and synthesis of **9***

Aldehyde **8** (15.0 mg, 0.039 mmol) was added to a cold (0°C) solution of NaB^3H_4 (1.8 mCi, 255 mCi/mmol) in THF/MeOH (4 ml, 1 : 1). The reaction mixture was stirred for 20 min at which time unlabeled $NaBH_4$ (1.5 mg, 0.040 mmol) was added to the reaction mixture (stirred for 15 min). The reaction was quenched by addition of saturated NH_4Cl and extracted with Et_2O (3×2 ml). Phases were separated and the organic layer was washed with sat. NaCl, and dried over Na_2SO_4. Removal of solvent under reduced pressure gave the corresponding allylic alcohol (13.0 mg, 93%) which was used without further purification. After removal of solvent under reduced pressure, the crude residue was dissolved in CH_2Cl_2 (2 ml), freshly prepared MnO_2[40] (58.0 mg, 20 eq., 0.680 mmol) was added at 0°C, and the reaction was stirred for 5 h. The suspension was filtered through a pad of celite, washed with CH_2Cl_2 (1×2 ml), and concentrated under reduced pressure followed by chromatographic purification over silica gel (5% ethyl acetate in hexanes) to yield pure aldehyde **9** (9.6 mg, 73%). The latter yield refers to product obtained from non-radioactive syntheses. The radioactive material was used without purification following extraction and removal of CH_2Cl_2 in the next step and the amount isolated was assumed to be the same as for the non-radioactive reaction.

*2,6,11,15-Tetramethyl-17-(2,6,6-trimethyl-cyclohex-1-enyl)-heptadeca-2,4,6,8,10,12,14,16-octaenoic acid ethyl ester (**10**)*

(Carbethoxyethylidene)triphenylphosphorane (18.0 mg, 0.050 mmol) was added to a solution of **9** (9.6 mg, 0.025 mmol) in THF (3.5 ml) and the reaction was refluxed for 5 h. The reaction mixture was diluted with water (4 ml) and extracted with Et_2O (3×2 ml). The combined ether extracts were dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by flushing it

through a small pad of silica in a Pasteur pipet (5% ethyl acetate in hexane) to yield ethyl ester **10** (9.4 mg, 80%). During the preparation of the radioactive material, the crude product was also flushed through a small pad of silica, however, it was not isolated and characterized, but was used directly in the next step.

UV: λ_{max} (hexanes) 440 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (1H, d, J= 11.6 Hz), 6.59 (4H, m), 6.4 (2H, m), 6.25 (1H, d, J= 11.1 Hz), 6.4 (4H, m), 4.21 (2H, q, J= 7.2 Hz), 2.00 (2H, m, CH_2), 1.97 (3H, s, CH_3), 1.95 (3H, s, CH_3), 1.69 (6H, s, 2 × CH_3), 1.60 (2H, m, CH_2), 1.54 (3H, s, CH_3), 1.45 (2H, m, CH_2), 1.29 (3H, *t*, J= 7.2 Hz, OCH_2 $\underline{CH_3}$), 1.00 (6H, s, 2 × CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.52, 143.84, 138.74, 137.83, 137.80, 137.65, 136.94, 136.53, 135.71, 135.39, 131.99, 131.91, 130.67, 129.48, 129.32, 126.95, 126.15, 125.72, 123.14, 60.46, 39.58, 34.22, 33.07, 29.65, 28.92, 21.73, 19.19, 14.32, 12.86, 12.81, 12.75, 12.68.

*[10′-^{3}H]-8′-apo-β-carotenoic acid (**3**)*

KOH (25%, 2 ml) was added to a solution of **10** (9.4 mg, 0.020 mmol), in iso-butyl alcohol (3 ml). The resulting mixture was heated at 95°C for 2 h and cooled to room temperature. H_2SO_4 (20%, 2 ml) was added and the reaction mixture was again heated to 70°C for 30 min. The organic layer was separated and washed with hot water (3 × 1 ml). Dichloromethane (5 ml) was added to the extracted iso-butyl alcohol resulting in two layers; the desired acid was extracted into CH_2Cl_2. The CH_2Cl_2 layer was separated, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified through a small pad of silica in a Pasteur pipet (5–15% ethyl acetate in hexane) to yield pure acid **3** (7.0 mg, 80%, 21 mCi/mmol, 0.34 mCi, 19% of total radionuclei incorporated) based on comparative TLC, and UV with authentic cold material, and all spectroscopic data were consistent with reported values.[41]

UV: λ_{max} (acetone) 440 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.20 (1H, bs,–CO_2H), 7.20 (1H, d, J= 10.8 Hz), 6.80–6.16 (11H, m, olefinic), 2.00 (2H, m, CH_2), 1.96 (6H, s, 2 × CH_3), 1.95 (3H, s, CH_3), 1.92 (3H, s, CH_3), 1.69 (3H, s, CH_3), 1.58 (2H, m, CH_2), 1.44 (2H, m, CH_2), 1.02 (6H, s, 2 × CH_3).

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