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Synthesis and preliminary biological evaluation of β-carotene and retinoic acid oxidation products

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Abstract—Synthesis of the β-carotene oxidation product, 2,3-dihydro-5,8-endoperoxy-β-*apo*-carotene-13-one (**1**) was achieved in six steps starting from β-ionone. Photo-oxygenation of all *trans*-retinoic acid (**8**) and 13-*cis*-retinoic acid (**9**) produced a mixture of 5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**10**) and 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**11**). Methylation of the crude photo-oxygenation mixture afforded the corresponding methyl esters **12** and **13**, respectively, both of which underwent ready aerial oxidation yielding hitherto unknown oxidation products of retinoic acid identified as methyl 5*S**,8*S**-epidioxy-9,10β-epoxy-5,8,9,10-tetrahydroretinoate (**14**) and methyl 13-*cis*-5*S**,8*S**-epidioxy-9,10β-epoxy-5,8,9,10-tetrahydroretinoate (**15**). Evaluation of **1**, all *trans*-retinoic acid (**8**), 13-*cis*-retinoic acid (**9**), and the photo-oxygenation products **10**–**15** in a panel of five cancer cell lines showed **1** to be inactive and that **11** is significantly cytotoxic compared with the other retinoic acid analogs suggesting the requirement of the carboxylic acid moiety and the *cis*-geometry of the 13(14) double bond for cytotoxic activity.

1. Introduction

Despite reported anticancer activity of β-carotene,[1] and epidemiological evidence that the consumption of β-carotene-rich fruits and vegetables is associated with lowered risk of cancer and heart disease,[2] its supplementation in three large clinical trials was found to be ineffective in protecting smokers and asbestos workers against lung cancer.[3] To explain this paradox, Omaye et al. have suggested that the relatively high partial oxygen pressure in lungs in the presence of reactive oxygen species produced from tobacco smoke and/or asbestos caused auto-oxidation of β-carotene.[4] In support of this hypothesis it has been reported that high dose supplementation of β-carotene activates a carcinogen-metabolizing enzyme of the P_{450} family in the lungs and enhances cell transformation of BALB/c 3T3 cells in the presence of cigarette smoke condensate.[5] Thus, some biological actions previously attributed to β-carotene may have been due at least in part due to its oxidation products.[6]

Carotenoids are known to directly interact with some free radical species in vitro,[7] and this may account for their radical scavenging properties. β-Carotene is employed clinically to prevent photosensitized tissue damage in humans with erythropoietic porphyria,[8] and may produce oxidation products on reaction with superoxide radicals.[9] We have previously reported that an enriched chromatographic fraction obtained from the oxidation of β-carotene with *m*-CPBA (*meta*-chloroperbenzoic acid) was capable of inhibiting DNA synthesis and arresting cells in G_0/G_1.[10] BHT (2,6-di-*tert*-butyl-4-methylphenol) protected against inhibition of growth of MCF-7 cells by β-carotene but not by this fraction suggesting that β-carotene oxidation products were responsible for inhibition of cell growth. This fraction also inhibited cholesterol synthesis, and pretreatment of MCF-7 cells with mevalonate, the product of the rate-limiting step of cholesterol synthesis, prevented inhibition of growth by oxidation products. The above enriched fraction neither bound nor inhibited binding of retinoic acid (RA) to its receptors (RAR, RXR, and PPAR). The major constituent of this minor frac-

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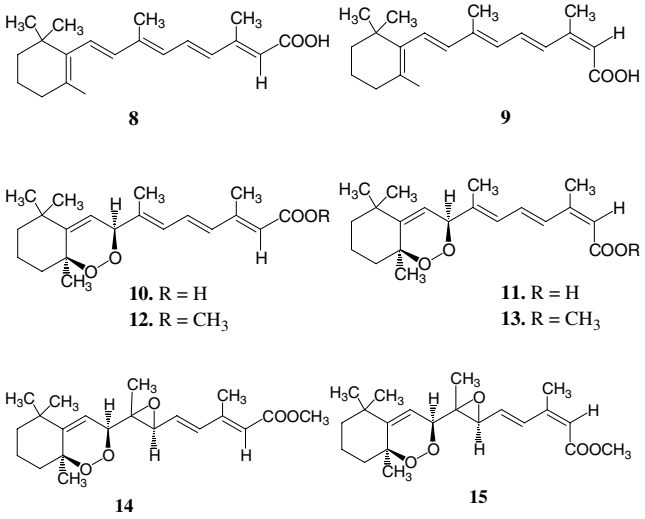
tion accounting for 70% by mass was characterized as 2,3-dihydro-apo-β-caroten-13-one-5,8-endoperoxide (DACE) (**1**).[10] It was of interest therefore, to synthesize **1** for confirmation of the proposed structure and evaluation of its biological activity.

Physiological oxidation of β-carotene is known to produce a number of retinoid acid-like compounds with potential biological activity.[11] Naturally occurring retinoids and their synthetic analogs play a key role in differentiation, proliferation, and apoptosis, and their use/potential in oncology, dermatology, and a variety of diseases are well documented.[12] RA (**8**) and its isomers, and their analogues are employed as chemotherapeutic agents in the treatment of many types of cancers including those of the liver,[13] ovary,[14] and breast.[15] Orally administered RA has been employed as a therapy for acute promyelocytic leukemia.[16] It is also used as a treatment of acne and psoriasis.[17] Retinoids are required for embryonic development,[18] epithelial cell growth and differentiation,[17] and vision.[19] They also exhibit anti-inflammatory properties[20] and suppress cytokine-induced production of NO in several cell types.[21] Oxidation of RA has led to the formation of number of oxygenation products including epoxides, dioxetanes, endoperoxides, and double bond cleavage products.[22] However, their biological actions have not been investigated. Herein we report the first synthesis of **1** and preparation of endoperoxides of RA and 13-*cis*-RA, **10** and **11**, their methyl esters **12** and **13**, and two novel epoxy-endoperoxide analogs of RA, **14** and **15**, and evaluation of their cytotoxic activity against a panel of five cancer cell lines.

2. Results and discussion

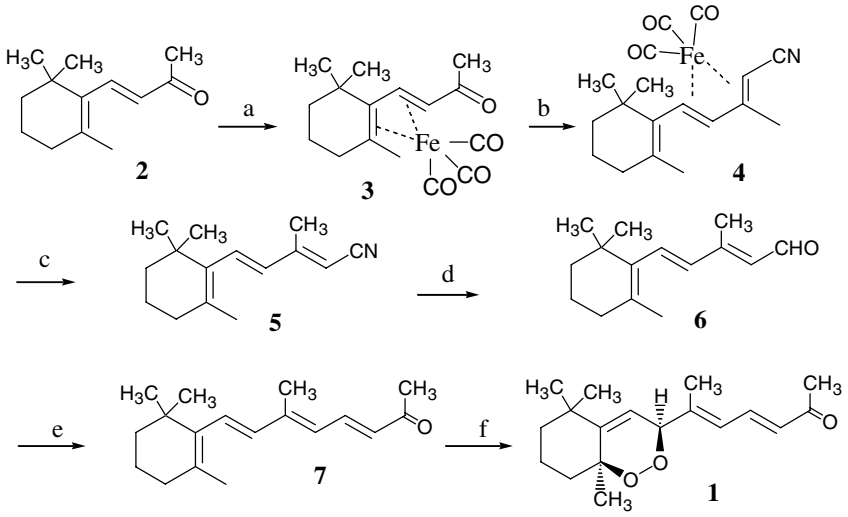
We envisioned a stepwise synthesis of DACE (**1**) starting from commercially available β-ionone (**2**). β-Ionone was converted into the aldehyde **6** via the tricarbonyliron derivative of the acetonitrile addition product **4** by employing the method reported by Wade et al.[23] The Wittig reaction of the aldehyde **6** with 1-(triphenyl-phosphoranylidene)-2-propanone gave **7** which on reaction with singlet oxygen afforded DACE (**1**) with ^{1}H and ^{13}C NMR data identical with those reported previously for this compound.[10] However, when evaluated for cytotoxic activity using HeLa and MCF-7 cancer cell lines the synthetic DACE was found to be inactive suggesting that the previously reported biological activity of the enriched fraction obtained by β-carotene oxidation may be due to a reaction product of DACE or a minor component present in this fraction. We are continuing our studies to isolate and characterize the compound(s) responsible for promising biological activity exhibited by this fraction of β-carotene oxidation product mixture (see Scheme 1).

We next turned our attention to the preparation of oxidation products of RA (**8**) and 13-*cis*-RA (**9**) for biological evaluation. Photo-oxygenation was carried out by bubbling oxygen through an ethanolic solution of **8** containing rose bengal during irradiation with UV light.[24] After disappearance of the starting material, solvent was removed under reduced pressure to give a crude product mixture, HPLC analysis of which indicated the presence of two major products. Chromatographic separation of a portion of this mixture yielded 5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**10**) and 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**11**).[25]



The remaining portion of the mixture of crude RA oxygenation products was methylated with CH_3I/K_2CO_3 to yield methyl 5*S**,8*S**-epidioxy-5,8-dihydroretinoate (**12**) and methyl 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoate (**13**).[13] Both **12** and **13** on exposure to air resulted in the formation of their novel derivatives, methyl 5*S**,8*S**-epidioxy-9,10β-epoxy-5,8,9,10-tetrahydroretinoate (**14**) and 13-*cis*-5*S**,8*S**-epidioxy-9,10β-epoxy-5,8,9,10-tetrahydroretinoate (**15**), respectively, the structures of which were elucidated by detailed analysis of their MS and NMR (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HSQC, and HMBC) spectral data. In particular its mass spectra showed the presence of an additional oxygen atom when compared with their precursors **12** and **13**. ^{1}H NMR analyses of these auto-oxidation products also indicated the absence of one olefinic proton and showed upfield shift of 9-CH_3 and 10-H suggesting that the newly introduced oxygen atom is attached to C-9 and C-10 to form an epoxy ring. Photo-oxygenation of 13-*cis*-RA (**9**) as for **8** also resulted in the formation of a mixture of 5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**10**) and 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoic acid (**11**) as major products suggesting that the oxygenation reaction of RA proceeds through a radical mechanism.

Synthetic DACE (**1**), RA (**8**), 13-*cis*-RA (**9**), and the photo-oxygenation products **10** and **11**, and their derivatives **12**–**15** were tested in a panel of five cancer cell lines [NCI-H460 (non-small cell lung), MCF-7 (breast), SF-268 (CNS glioma), MIA Pa Ca-2 (pancreatic carcinoma), and HeLa (human cervical carcinoma)] using the MTT assay.[26] Of these, only compounds **10** and **11** were found to be active at the concentrations tested (up to 30 μM) and **11** was significantly more cytotoxic than **10** (Table 1). Interestingly, all methylated com-



Scheme 1. Reagents and conditions: (a) $Fe_3(CO)_{12}$, toluene, N_2, 85 °C, 24 h, 42%; (b) LDA, THF, −70 °C and then CH_3CN, 30 min, 93%; (c) $CuCl_2$, EtOH, 25 °C, 6 h, 84%; (d) DIBAL, CH_2Cl_2, 0 °C, 3 h, 74%; (e) $Ph_3P{=}CH{-}C(O)CH_3$, toluene, 100 °C, N_2, 48 h, 50%; (f) O_2, toluene, rhodamine B, UV, 1 h (TLC control), 5%.

Table 1. Cytotoxicities (IC_{50}) of RA oxidation products **10** and **11** against a panel of five cancer cell lines[a]

Cell line[b]	NCI-H460	MCF-7	SF-268	MIA Pa Ca-2	HeLa
10	21.0	21.9	NA	13.9	19.8
11	2.1	2.7	3.5	1.7	2.0
Dox	0.01	0.07	0.04	0.05	ND

[a] Results are expressed as IC_{50} values in μM; NA, not active at 30 μM; ND, not determined.

[b] Key: NCI-H460, human non-small cell lung cancer; MCF-7, human breast cancer; SF-268, human CNS cancer (glioma); MIA Pa Ca-2, metastatic human pancreatic cancer; HeLa, human cervical carcinoma. Doxorubicin (Dox) was used as the positive control.

pounds were found to be inactive in the MTT assay. This suggests that the carboxylic acid moiety is required for cytotoxicity. Our findings further suggest the dependence of potential anticancer activity of retinoic acid analogs on the geometrical disposition of the 13(14)-double bond.

3. Experimental

3.1. General procedures

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and solvents for HPLC analysis were from VWR Scientific (San Francisco, CA). Analytical HPLC was performed on a Hitachi instrument equipped with L-6200A intelligent pump, L-4500 diode array detector, and P-6000 interface utilizing Hitachi Model D-7000 chromatography data station software using a Kromasil 5 μm C-18 column (4.6× 250 mm). Injections (15 μL) were made with AS-4000 intelligent auto sampler. The mobile phase consisted of methanol/water/formic acid (80:19.75:0.25) with a flow rate of 0.8 mL min^{-1}. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO Dip-370 polarimeter using $CHCl_3$ as solvent. IR spectra for KBr discs were recorded on a Shimadzu FTIR-8300 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$, acetone-d_6 and DMSO-d_6 and using residual solvents as internal standards with a Bruker DRX-500 instrument at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. Low resolution and high resolution MS were recorded, respectively, on Shimadzu LCMS QP8000α and JEOL HX110A spectrometers. The tetrazolium-based colorimetric assay (MTT assay)[26] was used for the in vitro assay of cytotoxicity to human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), human glioma (SF-268), human pancreatic carcinoma (MIA Pa Ca-2), and human cervical cancer (HeLa).

3.2. Synthesis of 2,3-dihydro-*apo*-β-carotene-13-one-5,8-endoperoxide (DACE) (1)

3.2.1. Preparation of 3. A mixture of β-ionone (1.0 g, 5.2 mmol) and dodecacarbonyltriiron (5.236 g, 10.4 mmol) in toluene (40 mL) was heated at 85 °C under an atmosphere of N_2 for 24 h. The reaction mixture was filtered through a column of neutral alumina to remove excess reagent and toluene was removed under reduced pressure. Purification of the crude product thus obtained by silica gel CC (eluant: 0.2% ether in hexane) afforded **3** (0.72 g, 52%).

3.2.2. Conversion of 3 to 4. To a stirred solution of LDA, prepared from *n*-BuLi (1.6 M hexane solution, 2.23 mL, 3.57 mmol) and diisopropylamine (0.508 mL, 3.57 mmol) in anhydrous THF (12 mL) was added a solution of CH_3CN (0.1866 mL, 3.57 mmol) in anhydrous THF (2 mL) at −70 °C and the resulting mixture was stirred for an additional 30 min. A solution of **3** (0.3963 g, 1.191 mmol) in anhydrous THF (4 mL) was then added to it at −70 °C and the mixture was allowed to warm up

to −50 °C over 1 h period. The reaction mixture was quenched with ice-cold water (1 mL) and extracted with ether (3× 50 mL). The combined ether layer was washed with water, dried (Na_2SO_4), evaporated, and the crude product purified by silica gel CC to yield **4** (0.39 g, 93%).

3.2.3. Conversion of 4 to 5. To a stirred solution of **4** (0.39 g) in EtOH (10 mL) at 25 °C was added a solution of $CuCl_2$ (0.785 g) in EtOH (15 mL). After 6 h, EtOH was removed under reduced pressure and the resulting residue was extracted with diethyl ether (3× 50 mL). The combined ether layers were washed with water, dried (Na_2SO_4), evaporated, and purified by silica gel CC to obtain **5** (0.20 g, 84%).

3.2.4. Conversion of 5 to (2E,4E)-3-methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (6). DIBALH (1.0 M solution in CH_2Cl_2, 0.95 mL) was added dropwise to a stirred solution of the nitrile **5** (0.2 g) in dry CH_2Cl_2 (5 mL) at 0 °C. After 3 h at 0 °C, excess DIBALH was destroyed by the addition of moist silica gel (5 mg + a drop of water), filtered, and the filtrate dried over Na_2SO_4. Evaporation and purification on silica gel CC afforded the aldehyde **6** (0.15 g, 74%).

3.2.5. Conversion of aldehyde 6 to 2,3-dihydro-*apo*-β-caroten-13-one (7). The phosphorane **6** [1-(triphenylphosphoranylidene)-2-propanone] (79.5 mg, 0.25 mmol) was added to a stirred solution of the aldehyde **6** (43.6 mg, 0.20 mmol) in dry toluene (5 mL) and heated under an atmosphere of N_2 at 100 °C for 48 h. Evaporation of toluene under reduced pressure followed by purification on silica gel CC (eluant: 1% EtOAc in hexane) afforded **7** as a yellow oil (26 mg, 50%).

3.2.6. Conversion of 7 to DACE (1). Oxygen gas was bubbled through a stirred solution of **7** (20 mg, 0.0774 mmol) in dry toluene containing rhodamine B (10% solution in EtOH, 1 drop) and the reaction mixture was irradiated under UV light. After 1 h (TLC control for the disappearance of **7**) the crude reaction mixture was passed through short bed of silica gel, evaporated under reduced pressure, and purified by prep. TLC (eluant: 10% EtOAc in hexane) to yield **1** (1.3 mg, 5%).

3.3. Photo-oxygenation of RA (8) and isolation of products

To a solution of RA (1.0 g, 3.33 mmol) in absolute EtOH (300 mL) was added a solution of rose bengal (35 mg, 34.4 μmol) in ethanol (200 mL). This mixture was then transferred to the photo-oxygenation apparatus and continuously oxygenated by bubbling oxygen gas through the solution while irradiating with UV light. The temperature of the reaction mixture was maintained below 10 °C by passing cool water through the cell jacket. After 70 min (TLC control for the disappearance of **8**), solvent was removed under reduced pressure to give the crude product mixture (1.14 g). Analysis of the crude mixture by HPLC indicated that it contained two major products. A portion (1.0 g) of the crude product was chromatographed over a column of silica gel (50 g) made up in hexane/CH_2Cl_2 (1:1) and eluted with CH_2Cl_2 followed by CH_2Cl_2 containing increasing amounts of methanol. Fractions eluted with CH_2Cl_2 were crystallized from hexane/CH_2Cl_2 to yield 5β,8β-epidioxy-5,8-dihydroretinoic acid (**10**) as a semisolid (450 mg, 41%). Fractions eluted with 1% MeOH in CH_2Cl_2 and 2% MeOH in CH_2Cl_2 were combined and washed with MeOH to give 13-*cis*-5β,8β-epidioxy-5,8-dihydroretinoic acid (**11**) as a white solid (167.2 mg, 15%).

3.3.1. 5*S,8*S**-Epidioxy-5,8-dihydroretinoic acid (10).** White semisolid; $[\alpha]_D^{25}$ −4.0° (c, 2.0, $CHCl_3$); NMR spectral data were identical with those reported previously.[25]

3.3.2. 13-*Cis*-5*S,8*S**-epidioxy-5,8-dihydroretinoic acid (11).** White solid, mp dec >151 °C; $[\alpha]_D^{25}$ 1.7° (c, 2.0, MeOH), IR (KBr) ν_{max} 2935, 1681, 1596, 1450, 1365, 1292, 1265, 1191, 1037, 964 cm^{-1}, ^1H NMR (acetone-d_6) δ 7.79 (1 H, d, J = 15.5 Hz, H-12), 6.97 (1H, dd, J = 15.5, 11.8 Hz, H-11), 6.23 (1 H, d, J = 11.8 Hz, H-10), 5.70 (1 H, s, H-14), 5.66 (1H, d, J = 3.7 Hz, H-7), 4.72 (1 H, d, J = 3.7 Hz, H-8), 2.08(3H, d, J = 0.8 Hz, 13-CH_3), 1.93 (3H, s, 9-CH_3), 1.56 (3H, s, 5-CH_3), 1.17 (3H, s, 1-CH_3), 1.12 (3H, s, 1-CH_3); APCIMS +ve mode m/z335 $[M+1]^+$.

3.4. Methyl 5*S**,8*S**-epidioxy-5,8-dihydroretinoate (12) and methyl 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoate (13)

To a solution of the endoperoxide mixture (2.2 g) in acetone (10 mL) were added K_2CO_3 (4 g) and CH_3I (5 mL) and stirred at 25 °C for 3 h. Reaction mixture was then filtered and filtrate was evaporated under reduced pressure to give a mixture of methyl esters (2.38 g). A portion (2.15 g) of this mixture was chromatographed over a column of silica gel (50 g) made up in 2% MeOH in CH_2Cl_2 and eluted with CH_2Cl_2 containing increasing amounts of MeOH. Twenty milliliters of fractions were collected and fractions with similar TLC profiles were combined to give 19 fractions. Repeated chromatography of the fractions eluted with 30% MeOH in CH_2Cl_2 yielded methyl 5*S**,8*S**-epidioxy-5,8-dihydroretinoate (**12**, 430 mg) and methyl 13-*cis*-5*S**,8*S**-epidioxy-5,8-dihydroretinoate (**13**, 235 mg) as gummy substances. ^1H NMR data of **12** and **13** were found to be identical with those reported previously for these two compounds.[25]

3.5. Methyl 5*S**,8*S**-epidioxy-9,10β-epoxy-5,8-9,10-tetrahydroretinoate (14)

Compound **12** (35 mg) was allowed to undergo aerial oxidation for 24 h at 25 °C. The major product was purified by prep. TLC (silica gel) to give **14** (8.2 mg,) as a colorless gum: ^1H NMR (acetone-d_6) δ 6.54 (1H, d, J = 15.7 Hz, H-12), 6.10 (1H, dd, J = 15.7, 7.2 Hz, H-11), 5.85 (1H, s, H-14), 5.54 (1H, d, J = 3.9 Hz, H-7), 4.25 (1H, d, J = 3.9 Hz, H-8), 3.65 (3H, s, OCH_3), 3.54 (1H, d, J = 7.2 Hz, H-10), 1.54 (3H, s, 5-CH_3), 1.37 (3H, s, 9-CH_3), 1.11 (3H, s, 1-CH_3), 1.10 (3H, s, 1-CH_3); HRFABMS m/z 363.4756 $[M+H]^+$ (calcd for $C_{21}H_{31}O_5$, 363.4741.

3.6. Methyl 13-*cis*-5*S**,8*S**-epidioxy-9,10β-epoxy-5,8-9,10-tetrahydroretinoate (15)

Compound **13** (11.2 mg) was allowed to undergo aerial oxidation for 48 h at 25 °C. The major product was purified by prep. TLC (silica gel) to give **15** (1.8 mg) as a colorless gum: ^{1}H NMR (acetone-d_6) δ 7.92 (1H, d, J = 16.0 Hz, H-12), 6.10 (1H, dd, J = 16.0, 7.4 Hz, H-11), 5.74(1H, s, H-14), 5.56 (1H, d, J = 3.9 Hz, H-7), 4.26 (1H, d, J = 3.9 Hz, H-8), 3.65 (3H, s, OCH_3), 3.55 (1H, d, J = 7.4 Hz, H-10), 1.78 (2H, m, H-3a, H-3b), 1.67 (1H, ddd, J = 14.0, 7.1, 3.4 Hz, H-4a), 1.58 (1H, ddd, J = 13.4, 5.2, 1.9 Hz, H-2a), 1.30 (1H, dd, J = 13.4, 3.7 Hz, H-2b), 1.20 (1H, dd, J = 14.0, 4.7 Hz, H-4b), 1.55 (3H, s, 5-CH_3), 1.38 (3H, s, 9-CH_3), 1.13 (3H, s, 1-CH_3), 1.11 (3H, s, 1-CH_3); ^{13}C NMR (acetone-d_6) δ 166.6 (C-15), 150.5 (C-13), 133.1 (C-11), 132.5 (C-12), 118.3 (C-14), 114.7 (C-7), 114.6 (C-6), 83.3 (C-8), 80.2 (C-5), 64.7 (C-9), 60.7 (C-10), 51.2 (OCH_3), 41.6 (C-2), 36.3 (C-4), 35.9 (C-1), 28.1 (1-CH_3), 25.9 (1-CH_3), 20.8 (9-CH_3), 19.7 (13-CH_3); HRFABMS m/z 363.4756 $[M+H]^+$ (calcd for $C_{21}H_{31}O_5$, 363.4741).

3.7. Photo-oxygenation of 13-*cis* retinoic acid (9)

To a solution of 13-*cis* RA (**9**, 200 mg, 666 μmol) in absolute ethanol (30 mL) was added a solution of rose bengal (35 mg, 6.9 μmol) in EtOH (20 mL). This mixture was oxygenated as for RA above. After 90 min (TLC control for the disappearance of **9**), solvent was removed under reduced pressure. The HPLC profile of the product mixture was found to be same as the HPLC profile of the products mixture obtained from the reaction with RA (**8**) above.

3.8. Cytotoxicity assays

The in vitro tetrazolium-based colorimetric assay (MTT assay) was used to measure inhibition of proliferation of human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), human glioma (SF-268), human pancreatic carcinoma (MIA Pa Ca-2), and human cervical carcinoma (HeLa) as previously reported.[26] All samples for assays were dissolved in DMSO.

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