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A facile synthesis of 1-ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one, a selective 15-lipoxygenase inhibitor

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Abstract—A facile method to synthesize 1-ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one, in yield of 92%, which showed selective inhibition effect on 15-lipoxygenase(soybean source) at $IC_{50} = 24.2 \pm 2.7$ μM while no inhibition effect was observed at greater than 300 μM on 5-lipoxygenase, lipid peroxidase, phospholipase A_2, protein kinase C, and cyclooxygenase.

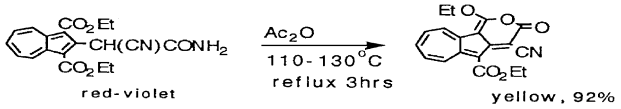
Lipoxygenase (LOX) is a biological target for many diseases such as asthma, atherosclerosis, and cancer.[1,2] LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation, in particular, the reticulocyte-type 15-LOX and the human 5-LOX, are well characterized with respect to their structural and functional properties.[3,4] Some natural azulene derivatives (chamazulene, guaiazulene) and synthetic azulene derivatives showed anti-oxidant activity and TXA_2/prostaglandin endoperoxide receptor antagonist.[5,6] Furthermore, synthetic azulene analogues such as 3-alkyl or 3-(hydroxy)alkylazulene-1-carboxylic acids and esters showed their effects on inhibition of soybean lipoxygenase by 100% at 1 mM.[7]

Diazoquinones (diazoxides, quinone diazides) are important synthetic intermediates because of their high reactivity, photochemically and thermochemically.[8] Among the 22 possible isomers of diazoazulenequinone (diazo-dihydro-oxoazulenes, diazoazulenequinones), the compounds of 2-D-2,6-AQ(2-diazo-2,6-azulenequinone) type are stable to be isolated while 6-D-2,6-AQ(6-diazo-2,6-azulenequinone) type are unstable to be isolated.[9] We had reported the facile synthesis of 2-diazo-1-3-dicyano-6-oxo-2,6-dihydroazulene and the diazotization of diethyl 6-amino-2-hydroxyazulene-1,3-dicarboxylate.[10,11] In this study, we report the synthesis of compounds of 1,2-azulenoquinone dimethide type, that is 1-ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one **2**, from tropolone,[9,12] via the corresponding diethyl 2-cyanocarbamoylmethylazulene-1,3-dicarboxylate **1**.[13,14]

A mixture of diethyl 2-chloroazulene-1,3-dicarboxylate,[12] (918 mg, 3 mmol) malonitrile (218 mg, 3.3 mmol), anhydrous 1,2-dimethoxy ethane (8 mL), sodium hydroxide 168 mg was stirred 24 h at 25 °C for 24 h to produce red color sodium salts precipitates. The mixture was then acidified by anhydrous acetic acid, then extracted with water/chloroform. The organic layer was neutralized with sodium bicarbonate and then worked up. The residue was chromatographed on silica-gel with successive elution (500 mL each) of benzene, chloroform, and ethyl acetate to obtain product diethyl 2-cyanocarbamoylmethylazulene-1,3-dicarboxylate **1**.[13,14]

Diethyl 2-cyanocarbamoylmethylazulene-1,3-dicarboxylate **1** (100 mg) and 3 mL acetic anhydride were used for reflux for 3 h at 110–130 °C to obtain (Scheme 1) orange-yellow needle crystal product **2**,[15] with a yield of 92% via a ring closure of the neighboring groups.

Assay of 5-lipoxygenase inhibition was run using a crude enzyme preparation from rat basophilic leukemia



Scheme 1. Acylation to form 1-ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one.

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cells (RBL-1). Test compound was pre-incubated with the enzyme for 5 min. The reaction was initiated by addition of substrate (linoleic acid), run for 15 min, pH 7.4, at 37 °C, and then terminated by addition of 0.3 M citric acid. The levels of 5-HETE (5-monohydroxyeicosatetraenoic acid) was determined by means of radioimmunoassay.[16–18]

Assay of 15-lipoxygenase (soybean source) inhibition was run using the enzyme pre-incubated with test compound for 4 min. The reaction was initiated upon the addition of substrate (linoleic acid), run for 10 min, pH 7.4, at 25 °C, and then terminated by the addition of NaOH. The formation of 15-HETE was determined by measuring absorbance at 234 nm.[19–23]

Lipid peroxidase (from rat liver) inhibition was assayed as the followings: Microsomes were prepared from rat livers and the protein concentration was determined. A reaction mixture consisting of 2 mg protein of the microsomal preparation, a NADPH generating system, with potent peroxidation antagonists and 20 mM CCl_4 was incubated for 12 min, pH 7.4, at 37 °C. The reaction was terminated by adding a mixture of thiobarbituric acid and trichloroacetic acid, and the absorbance was read at 535 nm. The latter was proportional to the concentration of malondialdehyde.[24]

Phospholipase A_2 (PLA_2) inhibition assay: PLA_2 (from porcine pancreas) was pre-incubated with test compound for 10 min in 0.1 M glycine–NaOH buffer, pH 9.0, at 37 °C. The reaction was initiated by addition of 0.03 μCi 1-palmitoyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine, run for 5 min pH 9.0, at 37 °C and then terminated by adding 0.2 M EDTA. [^{14}C]Oleate was extracted with acidic hexane, and an aliquot of the hexane layer was analyzed by scintillation counting. [25]

Protein kinase C (from rat brain) inhibition assay: the reaction mixture (0.2 mL) contained 5 mmol Tris–HCl, 50 mg of Histon III-S, 2.5 nmol of [γ-^{32}P]ATP, 2.5 mmol $MgCl_2$, with Triton X-100, phosphatidylserine, diacylglycerol. The reaction was initiated by addition of the enzyme, run for 15 min, pH 7.5, at 25 °C, and then terminated by 75 mM cold phosphoric acid. An aliquot was removed to spot on phosphocellulose paper, then counted to determine the radioactivity of ^{32}P-Histone.[26,27]

Cyclooxygenase inhibition assay: cyclooxygenase (from ram seminal vesicle) was incubated with arachidonic acid (500 μM) for 1.5 min at 27 °C in the presence or absence of test compounds. The reaction was run for 15 min, pH 7.5, at 27 °C, then terminated by adding trichloroacetic acid. Cyclooxygenase activity was determined by reading absorbance at 532 nm.[17,18,28,29]

Those starting, intermediate and product compounds including **1** and **2** were screened for their biological effects on the said enzyme inhibition assays. 1-Ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one **2** showed selective inhibition effect on 15-lipoxygenase at $IC_{50} = 24.2 \pm 2.7$ μM (phenidone was used as a reference compound in this 15-lipoxygenase inhibition assay and showed $IC_{50} = 2.2 \pm 0.2$ μM) while no inhibition effect was observed at greater than 300 μM on other enzymes, such as 5-lipoxygenase, lipid peroxidase, phospholipase A_2, protein kinase C, and cyclooxygenase.

1-Ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one **2**, containing the 1,2-azulenoquinone dimethide structure, showed yellow color which is different from the red-violet color of ordinary azulene compounds without 1,2-dimethide structure. By 2D NMR H–H COSY revealed that the five hydrogens on the seven-member ring were not replaced. In acid solvent CF_3COOH or CF_3COOD (TFA: trifluoroacetic acid), the UV λ_{max} of 1-ethoxy-4-cyano-5-ethoxycarbonyl-3*H*-azuleno[1,2-*c*]pyran-3-one **2** showed a strong absorbance at 477.5 nm which is quite different from that of being in neutral solvent MeOH, acetone, or $CHCl_3$. ^{1}H NMR spectra of various protons (H-6, 7, 8, 9, 10) of the compound also showed significant differences in neutral solvent if compared to that in TFA: Upfield chemical shifts with a difference of δ 0.13 (ppm) for H-7, 8, 9 (δ 8.11 in acetone-d_6 while δ 7.98 in CF_3COOD); with a difference of δ 0.63 (ppm) for H-6 (δ 9.34 in acetone-d_6 while δ 8.71 in CF_3COOD); and with a difference of δ 0.44 (ppm) for H-10 (δ 9.47 in acetone-d_6 while δ 9.03 in CF_3COOD). Normally a carbonium ion may cause downfield chemical shifts.[9] Therefore, it could be attested that it is not a 14 π resonance downfield chemical shifts 13C-*O*-12 π system (13 carbons and the one oxygen in the heterocycle participating in the resonance). Those observed upfield chemical shifts were the results of 13C–12 π resonance system. The protonation provided by TFA unto the lone pair electrons of the oxygen of the pyran ring produced an enlarged 13C-12 π resonance system (without the oxygen participating in the resonance)—an peripheral condensed system with paratropic character, an anti-aromaticity 13C–12 π resonance system. Whether this characteristics be related to the said biological effects and the related in vivo effects of compound **2** needs to be further explored.

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13. All melting points are uncorrected. IR spectra were recored on a Perkin-Elmer IR-983G spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker AC-300 spectrometer. Tetramethylsilane (TMS) was used as an internal standard for ^{1}H NMR. Chemical shifts (δ/ppm) and coupling constants (Hz) were measured with respect to TMS. Mass spectra were recorded on a Finnigan TSQ-46C spectrometer at 70 eV ionizing irradiation. Higher-resolution mass spectra (HRMS) were recorded on a JEOL JMS-HX 110 spectrometer. Ultraviolet-visible spectra were recorded on Shimadzu UV-202 and UV-160 spectrometer.
14. **1**: reddish violet needles (from benzene), mp 184–185 °C; yield 1.8%: UV: λ_{max} in MeOH nm (logε): 237 (4.48), 278 (4.44), 294$^{(sh)}$ (4.59), 304 (4.62), 366 (3.83), 510 (2.83), λ_{max} in MeOH–aq NaOH: 226 (4.27), 380 (4.40), 494 (4.13); IR (KBr, cm^{-1}): 3460, 3195, 2242 (w), 1704, 1684, 1431, 1198. ^{1}H NMR (60 MHz, DMSO-d_6) δ 1.56 (6H, t, J = 7.0 Hz, CH_3), 4.61 (4H, q, J = 7.0 Hz, CH_2), 6.46 (s, CH), 8.00 (3H, m, H-5, 6, 7), 9.81 (2H, d, J = 11 Hz, H-4, 8); Elem. anal. found: C, 64.68; H, 5.44; N, 7.91%; Calcd for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91%.
15. **2**: mp 172–173 °C, orange yellow needles from EtOH, UV λ_{max} nm (logε, MeOH): 235.2 (4.349), 276.2 (4.354), 301.2 (4.56), 334.6 (3.83), 363.6 (3.87), 390$^{(sh)}$ (3.23), 490 (2.98), 580$^{(sh)}$ (2.50). UV λ_{max} nm (logε, $CHCl_3$): 250.2 (4.32), 325$^{(sh)}$ (4.5), 340 (4.54), 410 (3.83), 430$^{(sh)}$ (3.33), 600 (2.12), 640$^{(sh)}$ (1.93), 680$^{(sh)}$ (1.78), 715$^{(sh)}$ (1.60), 760$^{(sh)}$ (1.56), 784$^{(sh)}$ (1.46). UV λ_{max} (logε, CF_3COOH): 270 (3.81), 290 (4.05), 305$^{(sh)}$ (4.27), 325.2 (4.5), 340 (4.5), 395$^{(sh)}$ (3.41), 445$^{(sh)}$ (3.70), 477.5 (3.95), 585$^{(sh)}$ (1.90), 610$^{(sh)}$ (1.76), 630$^{(sh)}$ (1.70), 710$^{(sh)}$ (1.51), 755 (1.46), 790 (1.40). ^{1}H NMR (acetone-d_6) (δ): 1.44 (3H, t, J = 7.3 Hz, –$OCH_2C\underline{H}_3$), 1.55 (3H, t, J = 7.3 Hz, –$OCH_2C\underline{H}_3$), 4.48 (2H, q, J = 6.8 Hz, –$OC\underline{H}_2CH_3$), 4.75 (2H, q, J = 6.7 Hz, –$OC\underline{H}_2CH_3$), 8.11 (3H, m, H-7, 8, 9), 9.34 (1H, dd, J = 1.2, 11.1 Hz, H-6), 9.47 (1H, dd, J = 1.3, 9.7 Hz, H-10); ^{1}H NMR (CF_3COOD) (δ): 1.69 (3H, t, J = 7.2 Hz, –$OCH_2C\underline{H}_3$), 1.84 (3H, t, J = 7.2 Hz, –$OCH_2C\underline{H}_3$), 4.69 (2H, q, J = 7.2 Hz, –$OC\underline{H}_2CH_3$), 5.18 (2H, q, J = 7.2 Hz, –$OC\underline{H}_2CH_3$), 7.98 (3H, m, H-7, 8, 9), 8.71 (1H, d, J = 11.6 Hz, H-6), 9.03 (1H, d, J = 7.4 Hz, H-10). ^{13}C NMR (δ): 14.32, 14.60, 60.57, 68.39, 79.05, 108.50, 114.51, 116.11, 134.53, 134.72, 136.58, 137.21, 140.84, 144.80, 148.11, 154.80, 168.13, 171.91, 202.27; IR (KBr, cm^{-1}): 2224, 1758, 1684, 1556, 1209, 1140, 1061, 1007. EIMS (20 eV) (m/z, %): 337 (M^+, 54), 281 (24), 237 (100), 193 (33), 164 (45), 151 (19), 101 (5), 55 (20); Elem. anal. found: C, 67.91; H, 4.79; N, 4.15%; Calcd for $C_{19}H_{15}NO_5$: C, 67.65; H, 4.48; N, 4.15%. HRMS: found M^+, 337.0933 (Calcd for $C_{19}H_{15}NO_5$: M^+, 337.0948).
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