Graphical abstract

Nitration reaction of lutein with peroxynitrite

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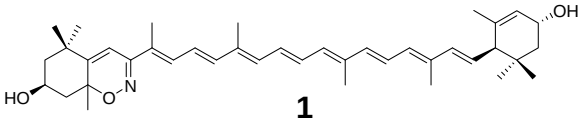
A novel lutein-6H-1,2-oxazine (**1**) along with 15-nitirolutein (**2**) and 15'-nitrolutein (**3**) were isolated from the products of the reaction of lutein with peroxynitrite.



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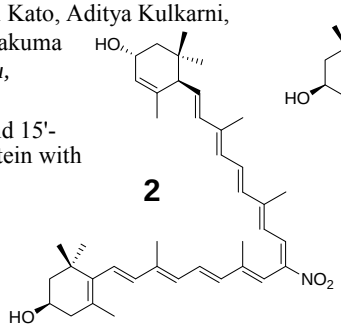

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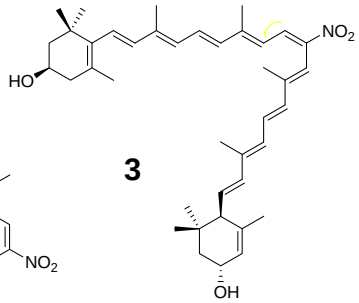

3

Nitration reaction of lutein with peroxynitrite

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Abstract---The in vitro reactivity of lutein toward peroxynitrite was investigated, and the reaction products produced by scavenging with peroxynitrite were analyzed. A novel lutein-6H-1,2-oxazine (**1**) along with 14-s-*cis*-15-nitirolutein (**2**) and 14'-s-*cis*-15'-nitrolutein (**3**) were isolated from the products of the reaction of lutein with peroxynitrite. These results indicate that lutein is able to capture peroxynitrite and nitrogen dioxide radicals from their molecules to form oxazine or nitrocarotenoids.

Keywords: lutein, reaction with peroxynitrite, lutein-6H-1,2-oxazine, 14-s-*cis*-15-nitrolutein, 14'-s-*cis*-15'-nitrolutein

Peroxynitrite, the reaction product of superoxide and nitric oxide, is a powerful oxidant produced by macrophages and neutrophils. Peroxynitrite is known to induce DNA strand scission, protein modification by nitration, and hydroxylation and lipid peroxydation in LDL. Previously, we first reported the formation of nitro-carotenoids by the reaction of β-carotene and astaxanthin with peroxynitrite. These results indicated that β-carotene and astaxanthin are able to capture peroxynitrite and nitrogen dioxide radicals from molecules to form nitro-carotenoids.[1,2] This information would be of value to those investigating the peroxinitrite scavenging action of carotenoids in *vivo*.

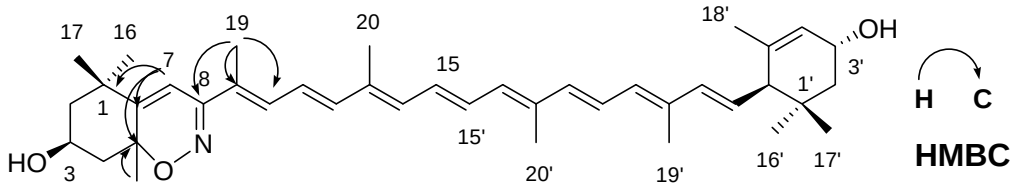
Lutein [(3*R*,3'*R*,6'*R*)-β,ε-carotene-3,3'-diol] and its metabolites, (3*R*,3'*S*:*meso*)-zeaxanthin and 3'-dehydrolutein, along with (3*R*,3'*R*)-zeaxanthin are presented in the macula and they perform a defense function against oxidation injury in the eyes. Lutein also prevents age related macular degeneration (AMD).

In the present study, we investigated the reaction of lutein with peroxynitrite because lutein has an asymmetric structure and so might provide some new reaction products by this reaction.

All-*trans*-lutein was reacted with peroxynitrite,[3,4)] and the reaction products were analyzed by HPLC.

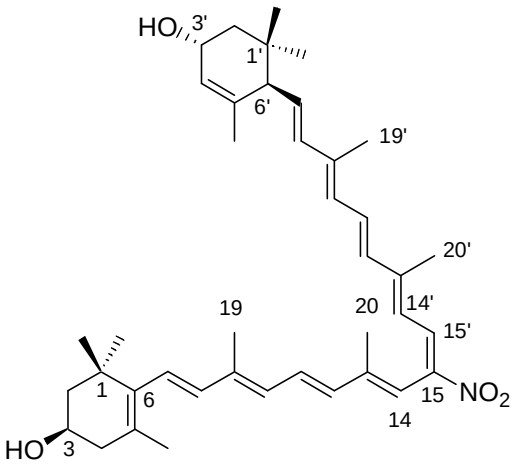
Compound **1**[5,6] (yield 1.5 mg) showed absorption maxima at 430, 457, and 486 nm. Acetylation of **1** gave a diacetate. Its molecular formula was determined to be $C_{40}H_{55}O_3N$

by HRFAB-MS, and it showed a structure lutein NO adduct. The ^{1}H and ^{13}C NMR signals of **1** were assigned by ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC experiments. The ^{13}C NMR signals at C-5, C-6, C-7, and C-8 were significantly different from those of lutein. The partial structure of C-5-C6=C7-C8 was elucidated from HMBC experiments. The chemical shift value of the quaternary carbon at C-5 (δ 80.1) indicated that an oxygen group was attached to C-5. On the other hand, the chemical shift value of the quaternary carbon at C-8 (δ 142.6) indicated that nitrogen was attached to C-8 by a double bond.[7] These spectral data were in agreement with the partial structure of –O-C5-C6=C7-C8=N-. From the HRMS data, oxygen was found to be bound to nitrogen by a single bond. Therefore, the partial structure of a 6-membered oxazine ring was elucidated. The remaining structural features were also confirmed by NOESY correlations between CH_3-16/17 and H-7, CH_3-19 and H-7/11, CH_3-20 and H-11/15, CH_3-16'/17' and H-7', CH_3-19' and H-7'/11', and CH_3-20' and H-11'/15'. The HMBC spectrum showed cross peaks at CH_3-16/17 to C-6, CH_3-18 to C-5/6, and CH_3-19 to C-8/9/10, indicating a 6-membered oxazine skeleton. Therefore, the structure of **1** was determined to be lutein-6H-1,2-oxazine. The formation mechanism of **1** might be assumed to be the direct reaction of lutein with peroxynitrite.



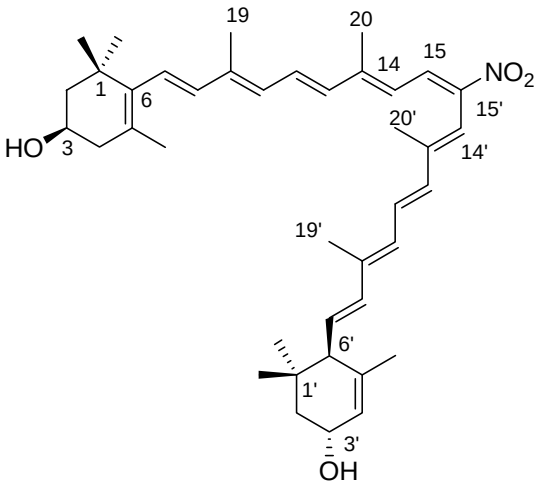
Lutein-6H-1,2-oxazine (1)

Compound **2**[8] (yield 3.5 mg) showed absorption maxima at 322, 465 nm. Its molecular formula was determined to be $C_{40}H_{55}O_4N$ by HRFAB-MS and it demonstrated a NO_2 substituted lutein structure. This structure was also characterized from ^{1}H and ^{13}C NMR including 2D NMR experiments. The partial structure of the end group and the polyene chain of compound **2** were characterized by ^{1}H NMR and ^{13}C NMR including ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC experiments. The downfield shift of the ^{13}C NMR signal at C-15 (δ 145.8, quaternary carbon) along with disappearance of a methylene proton at the C-15 position in ^{1}H NMR compared with lutein, clearly indicated that a nitro group was attached to the C-15 position of lutein. Furthermore, the change in the coupling pattern and the downfield shifts of the ^{1}H NMR signals at H-15' (δ 8.05) and H-14 (δ 6.19) compared with lutein, supported the substitution position of the nitro group at C-15. The steric structure was confirmed by NOESY correlations between CH_3-16/17 and H-7, CH_3-19 and H-7/11, CH_3-20 and H-11/14', CH_3-16'/17' and H-7', CH_3-19' and H-7'/11', and CH_3-20' and H-11'/15'. Spectral analysis of compound **2** indicated its structure to be 14-s-*cis*-15-nitrolutein (**2**).



14-s-*cis*-15-nitrolutein (2)

Compound **3**[9] (yield 3.2 mg) showed maxima at 343, 447 nm and molecular formula as those of **2**. The ^{1}H and ^{13}C NMR data of **3** were very similar to those of **2** except for at the 14, 15, 14', and 15' positions. The quaternary carbon at C-15' (δ 145.8) and doublet signal at H-15 (δ 8.06) clearly indicated that a nitro group was attached to C-15'. Its steric structure was confirmed by NOESY data. The final structure of compound **3** was established as 14'-s-*cis*-15'-nitrolutein (**3**).



14'-s-*cis*-15'-nitrolutein (3)

The versatility of the reaction mode is suggestive of the involvement of several

different active species in the reaction with peroxynitrite. There are still many unidentified products, the identification of which may provide additional new reaction modes for the reaction of peroxynitrite with carotenoids as well as various other biological antioxidation systems. These reactions would probably be found in *vivo* and contribute to the degradation of biological systems, eventually leading to pathogenic disease processes. Better understanding of the behavior of peroxynitrite toward a wide variety of biological antioxidation systems would enable us to predict the role of peroxynitrite in *vivo* and provide valuable information on its physiological significance.

Acknowledgements

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References and notes

1. Yoshioka, R.; Hayakawa, T.; Ishizuka, K.; Kulkarni, A.; Terada, Y.; Maoka, T.; Etoh, H. *Tetrahedron Lett.* **2006**, 47, 3637-3640.
2. Hayakawa, T.; Kulkarni, A.; Terada, Y.; Maoka, T.; Etoh, H. *Biosci. Biotechnol. Biochem.* **2008**, 72, 2716-2722.
3. Niwa, T.; Doi, U.; Kato, Y.; Osawa, T. *J. Agric. Food Chem.* **2001**, 49, 177-182.
4. All-*trans*-lutein (400 mg, from Kemin Health Asia) was dissolved in 50 mL of THF

(final concentration 5.7 mM). To this, TFA was added in order to make up the final concentration to 2%, before the addition of 16 mL of peroxynitrite (final concentration: 6.8 mM). Then the solution was allowed to react for 1 min. Then, to the above mixture, 300 mL of $CHCl_3$ and 300 mL of H_2O were added so as to separate the reaction products into organic and aqueous phases. The whole procedure was performed three times. The organic layer was dried over sodium sulfate and concentrated. This organic concentrate was then subjected to HPLC analysis using the Develosil C30-UG-5 (250 x 4.6 i.d. ; MeCN:H_2O = 82:18, flow rate: 1 min, column temp: 40^{o}C) column. A more specific separation procedure was performed using the Deverosil C30-UG-5 (250 x 4.6 i.d. mm; MeCN:H_2O = 75:25) column.

5. In HPLC analysis for lutein, four main groups of reaction products were observed, namely fractions A (*t*R 3-12 min), B (*t*R 12-28 min), C (*t*R 30-36 min), and D (*t*R 50-68 min). The peaks in fraction A were observed to have a lower λ max, indicating then to be apo-carotenals. The fraction B and C compounds, which contained the main reaction products, were observed to be oxygenated products with a C-40 skeleton. The compounds in fraction D were 9- and 9'-*cis*-lutein and 13- and 13'-*cis*-lutein. They were identified from their values in the literature; Khachike, F.; Englert, G.; Daitch, C. E., Beecher, G. R.; Lusby, W. R. J. *Chromatogr. Biomed. Appl*. **1992**, 582, 153-166. Further separation of fraction B gave compounds **2** and **3** and fraction C gave

compound **1**.

6. Lutein-6H-1,2-oxazine (**1**) UV-vis λ max (Et_2O) nm 430, 457, 486; HR-FAB MS 597.4173 (M^+, calc. for $C_{40}H_{55}O_3N$, 597.4182); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.85 (H_3-17', s), 1.00 (H_3-16', s), 1.23 ((H-4α, dd, *J*=13.0, 4.0), 1.25 (H-2α, overlapped), 1.26 (H_3-17, s), 1.31 (H_3-16, s), 1.37 (H-2'α, dd, *J*=13.0, 7.0), 1.60 (H_3-18, s), 1.62 (H_3-18', s), 1.85 (H-2'β, dd, *J*=13.0, 6.0), 1.91 (H_3-19', s), 1.98 (H_3-20', s), 1.99 (H_3-20, s), 2.02 (H-2β, ddd, *J*=13.0, 4.0, 2.0), 2.17 (H_3-19, s), 2.41(H-6', d, *J*=10.0), 2.53 (H-4 β, ddd, *J*=13.0, 4.0, 2.0), 4.22 (H-3, m), 4.25 (H-3', m), 5.44 (H-7', dd, *J*=15.5, 10.0), 5.55 (H-4', brs), 6.14 (H-8', d, *J*= 15.5), 6.14 (H-10', d, *J*= 11.0), 6.24 (H-7, s), 6.24 (H-12, d, *J*= 15.0), 6.25 (H-14', d, *J*= 11.0), 6.31 (H-14, d, *J*= 11.0), 6.36 (H-12', d, *J*= 15.0), 6.62 (H-11', dd, *J*=15.0, 11.0), 6.64 (H-15, m), 6.64 (H-15', m), 6.69 (H-11, dd, *J*=15.0, 11.0), 8.30 (H-10, d, *J*=11.0) ^{13}C-NMR ($CDCl_3$, 125MHz) δ 12.7 (C-20), 12.8 (C-20'), 13.1 (C-19'), 15.1 (C-19), 22.9 (C-18'), 23.4 (C-18), 24.2 (C-17'), 26.5 (C-17), 29.4 (C-16), 29.5 (C-16'), 34.0 (C-1'), 34.8 (C-1), 44.6 (C-2'), 45.9 (C-4), 51.4 (C-2), 54.9 (C-6'), 65.6 (C-3), 65.9 (C-3'), 80.1 (C-5), 116.1 (C-7), 124.5 (C-4'), 125.1 (C-11'), 128.3 (C-9), 128.8 (C-7'), 129.8 (C-15), 129.9 (C-11), 130.7 (C-15'), 130.8 (C-10'), 132.4 (C-12), 132.5 (C-13, 13'), 134.1 (C-10), 134.5 (C-14), 135.3 (C-9'), 137.1 (C-12', 14'), 137.7 (C-8'), 138.0 (C-5'), 142.6 (C-8), 156.3 (C-6); Acetylation of **1** with acetic anhydride in pyridine at room temperature for 1 hr gave a diacetate, which

showed molecular ion m/z 681 by FAB MS.

7. Kalinowski, H-O.; Berger, S.; Braun, S.; *Carbon-13 NMR spectroscopy*; John Wiley & Son Ltd; New York, 1988; p.243 and p.391.

8. 14-s-*cis*-15-Nitrolutein (**2**) UV-vis λ max (Et_2O) nm 322, 465; HR-FAB MS 613.4139 (M^+, calc. for $C_{40}H_{55}O_4N$, 613.4131); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.85 (H_3-16', s), 1.00 (H_3-17', s) 1.09 (H_3-16 and 17, 2s), 1.37 (H-2'α, dd, *J*=14.0, 7.0), 1.48 (H-2α, dd, *J*=12.0, 11.0), 1.62 (H_3-18', s), 1.75 (H_3-18, s), 1.77 (H_3-20, s), 1.77 (H-2β, ddd, *J*=12.0, 4.0, 1.5), 1.84 (H-2'β, dd, *J*=14.0, 6.0), 1.95 (H_3-19', s), 1.99 (H_3-19, s), 2.04 (H-4α, dd, *J*=18.0, 10.0), 2.16 (H_3-20', s), 2.40 (H-4β, ddd, *J*=18.0, 6.0, 1.5), 2.43 (H-6', d, *J*=10.0), 4.00 (H_3-3, m), 4.25 (H_3-3', m), 5.56 (H-4', s), 5.56 (H-7', dd, *J*=15.0, 10.0), 5.86 (H-14, d, *J*=11.0), 6.08 (H-10, d, 11.5), 6.08 (H-10', d, *J*=11.0), 6.10 (H-7, d, *J*=16.0), 6.16 (H-8, d, *J*=16.0), 6.16 (H-8', d, *J*=15.0), 6.19 (H-14, s), 6.40 (H-12', d, *J*=15.0), 6.50 (H-12, d, *J*=15.0), 6.79 (H-11, dd, *J*=15.0, 11.5), 6.90 (H-11', dd, *J*=15.0, 11.0), 8.05 (H-15', d, *J*=12.0) ^{13}C-NMR ($CDCl_3$, 125MHz) δ 13.1 (C-19), 13.3 (C-19'), 13.6 (C-20'), 15.3 (C-20), 20.5 (C-17), 22.9 (C-18, 18'), 24.3 (C-17'), 28.7 (C-16), 29.5 (C-16'), 34.0 (C-1'), 37.1 (C-1), 42.6 (C-4), 44.6 (C-2'), 48.4 (C-2), 54.9 (C-6'), 65.0 (C-3), 65.9 (C-3'), 118.9 (C-14), 124.6 (C-4'), 125.6 (C-14'), 126.8 (C-5, 7), 127.9 (C-11), 130.0(C-10'), 130.4 (C-11', 15'), 131.1 (C-7'), 131.2 (C-10), 135.7 (C-12), 136.1 (C-9', 12'), 137.6 (C-5', 6, 8'), 138.1 (C-9), 138.2 (C-8), 142.8 (C-13), 145.8

(C-15), 149.1 (C-13').

9. 14'-s-*cis*-15'-Nitrolutein (**3**) UV-vis λ max (Et_2O) nm 343, 447; HR-FAB MS 613.4139 (M^+, calc. for $C_{40}H_{55}O_4N$, 613.4131); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.86 (H_3-16', s), 1.10 (H_3-16, s) 1.08 (H_3-16 and 17 , 2s), 1.37 (H-2'α, dd, *J*=14.0, 7.0), 1.48 (H-2α, dd, *J*=12.0, 11.0), 1.64 (H_3-18', s), 1.74 (H_3-18, s), 1.76 (H_3-20', s), 1.77 (H-2β, ddd, *J*=12.0, 4.0, 1.5), 1.85 (H-2'β, dd, *J*=14.0, 6.0), 1.93 (H_3-19', s), 2.01 (H_3-19, s), 2.04 (H-4α, dd, *J*=18.0, 10.0), 2.16 (H_3-20, s), 2.40 (H-4β, ddd, *J*=18.0, 6.0, 1.5), 2.43 (H-6', d, *J*=10.0), 4.00 (H_3-3, m), 4.25 (H_3-3', m), 5.40 (H-7', dd, *J*=15.0, 10.0), 5.56 (H-4', s), 5.96 (H-14, d, *J*=12.0), 6.10 (H-10', d, *J*=11.0), 6.10 (H-7, d, *J*=16.0), 6.16 (H-8, d, *J*=16.0), 6.16 (H-10, d, *J*=11.5), 6.20 (H-8', d, *J*=15.0), 6.30 (H-14', s), 6.40 (H-12, d, *J*=15.0), 6.49 (H-12', d, *J*=15.0), 6.75 (H-11', dd, *J*=15.0, 11.0), 6.95 (H-11, dd, *J*=15.0, 11.5), 8.06 (H-15, d, *J*=12.0) ^{13}C-NMR ($CDCl_3$, 125MHz) δ 13.2 (C-19, 19'), 13.6 (C-20), 15.3 (C-20'), 20.5 (C-17), 21.6 (C-18), 22.9 (C-18'), 24.3 (C-17'), 28.7 (C-16), 29.5 (C-16'), 34.0 (C-1'), 37.1 (C-1), 42.6 (C-4), 44.6 (C-2'), 48.4 (C-2), 54.9 (C-6'), 65.0 (C-3), 65.9 (C-3'), 119.0 (C-14'), 124.6 (C-4'), 125.8 (C-14), 126.8 (C-5, 7), 127.8 (C-11', 12'), 130.1 (C-7', 10'), 130.2 (C-10), 130.4 (C-11, 15), 136.1 (C-9, 12), 137.6 (C-5', 6, 8'), 138.0 (C-8), 138.1 (C-9'), 142.8 (C-13'), 145.8 (C-15'), 149.1 (C-13); NOESY correlations between CH_3-16/17 and H-7, CH_3-19 and H-7/11, CH_3-20 and H-11/15, CH_3-16'/17' and H-7', CH_3-19' and H-7'/11', and CH_3-20' and H-14/11'.

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