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Original scientific paper

Anti-inflammatory activity of synthetic and natural glucoraphanin

QUAN V. VO[1,2]*, PHAM C. NAM[3]**, THUC N. DINH[4], ADAM MECHLER[5] and THI T. V. TRAN[6]

[1]*Department for Management of Science and Technology Development, Ton Duc Thang University, Ho Chi Minh City, Vietnam,* [2]*Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam,* [3]*Department of Chemical Engineering, University of Da Nang – University of Science and Technology, Vietnam,* [4]*Faculty of Natural Sciences, Hong Duc University, Thanh Hoa, Vietnam,* [5]*La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia and* [6]*Department of Chemistry, Hue University of Science, 77 Nguyen Hue, Hue, Vietnam*

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Abstract: Glucoraphanin is one of the best known glucosinolates because of its health benefits. The compound is known to eliminate carcinogens in tissue and hence is frequently studied for its cancer preventative properties. In this work, the total synthesis of *α*- and *β*-glucoraphanin epimers was attempted. *β*-Glucoraphanin potassium salt was successfully synthesized in high overall yield, whereas the *α*-epimer was found to be unstable as it decomposed in the final step of the total synthesis. The anti-inflammatory activity of the synthetic glucoraphanin was determined by inhibition of the release of tumor necrosis factor alpha (TNF-*α*) secretion in lipopolysaccharide-stimulated THP-1 cells. It was shown that in the presence of either the synthetic or natural glucoraphanin, TNF-*α* secretion was significantly reduced (≈52 % inhibition) at a concentration of 15 μM.

Keywords: broccoli; TNF-*α*; sulforaphane; glucosinolates; synthesis; THP-1.

INTRODUCTION

Glucoraphanin (4-methylsulfinylbutyl glucosinolate, GRP) is the most abundant glucosinolate in common culinary brassica species, such as broccoli (*Brassica oleracea*)[1] or hoary cress (*Cardaria draba*).[2] When ingested, it is hydrolyzed by the enzyme myrosinase to sulforaphane that imparts numerous health benefits: it was shown to eliminate carcinogens in living tissue[3,4] and it is an inducer of phase 2 enzymes (glutathione *S*-transferase and quinone reductase)[5] that are linked to cancer protection.[6,7] Consistently it is possible, and desirable,

*,** Corresponding authors. E-mail: (*)vovanquan@tdtu.edu.vn; (**)pcnam@dut.udn.vn
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to create a GRP-based nutraceutical that retains the health advantages of sulforaphane and natural GRP.

The evaluation of the biosafety and anti-tumor activity of purified and semi-purified GRP was reported previously.[8,9] The studies were performed on male F344 rats by testing the effect of GRP on ethoxyresorufin *O*-deethylase and/or NQO1 activity. The response to GRP was similar for the purified and semi-purified GRP preparations, suggesting that semi-purified preparations could be used in supplements and to fortify foods. However, there are no reports of control studies of GRP bioactivity using synthetic GRP that is a better defined source with much reduced and easily identifiable impurities. Furthermore, there are indications that the activity of GRP is more diverse and/or it exerts a protective function *via* an anti-inflammatory effect, similar to the pathway reported for aromatic and indole glucosinolates (GLs).[10,11] There is a link between chronic inflammation and carcinogenesis,[12–14] and thus a study of anti-inflammatory activity could be a simple and inexpensive step to initially evaluate the anticancer activity of glucoraphanin. Therefore, in this study, a comprehensive study of the total synthesis of α- and β-GRP potassium salts and the measurements of their anti-inflammatory activity in comparison with other typical glucosinolates are reported.

EXPERIMENTAL

General methods

Melting points (m.p.) were recorded on a hot stage apparatus and are uncorrected. Optical rotations were measured at the stated temperatures in the stated solvent on a polarimeter at the sodium d-line (589 nm); $[\alpha]_D$ values are given in 10^{-1}° cm^2 g^{-1}. Infrared spectra (ν_{max}) were recorded on a FT-IR spectrometer. The samples were analyzed as KBr discs (for solids) or as thin films on NaCl plates (for liquids/oils). Unless otherwise specified, the proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a 300 MHz spectrometer operating at 300 MHz for protons and 75 MHz for carbon nuclei. Chemical shifts were recorded as δ values in ppm. The spectra were acquired in deuterated chloroform ($CDCl_3$), methanol-d_4 (CD_3OD) or deuterium oxide (D_2O) at 300 K unless otherwise stated. For the ^{1}H-NMR spectra recorded in $CDCl_3$, CD_3OD and D_2O, the peaks due to residual $CHCl_3$, CD_3OD and D_2O (δ_H 7.24, 3.28 and 4.65 ppm, respectively) were used as the internal reference, while the central peaks (δ_C 77.0 and 49.0 ppm) of $CDCl_3$ and CD_3OD were used as the reference for the proton-decoupled ^{13}C-NMR spectra. Low-resolution mass spectra were measured on a mass spectrometer at 300 °C at a scan rate of 5500 *m*/*z* per s using water/methanol/acetic acid in a volume ratio of either 0/99/1 or 50/50/1 as the mobile phase. Accurate mass measurement was by mass spectrometry with a heated electrospray ionization (HESI) source. The mass spectrometer was operated with full scan (50–1000 amu) in the positive or negative FT mode (at a resolution of 100,000). The analyte was dissolved in water/methanol/acetic acid in a volume ratio of 0/99/1 or 50/50/1 and infused *via* syringe pump at a rate of 5 μL/min. The heated capillary was maintained at 320 °C with a source heater temperature of 350 °C and the sheath, auxiliary and sweep gases were at 40, 15 and 8 units, respectively. The source voltage was set to 4.2 kV. The solvents were dried over standard drying agents and freshly distilled before use. Ethyl acetate and hexane used for chromatography were distilled prior to use. All solvents

were purified by distillation. Reactions were monitored by thin layer chromatography (TLC) on silica gel 60 F_{254} plates with detection by ultra-violet (UV) fluorescence or charring with a basic potassium permanganate stain. Flash column chromatography was performed on silica gel 60 particle size 0.040–0.063 mm (230–400 mesh).

General procedures for the synthetic compounds

*1-Thio-*α*- or* β*-D-glucopyranoze 2,3,4,5-tetraacetate 1-[(1Z)-*N*-hydroxy-5-(methylsulfinyl)pentanimidate] (**2a** or **2b**)*. To a suspension of **1** (150 mg, 0.9 mmol) in DCM (10 mL) was added pyridine (0.09 mL, 0.95 mmol) and then *N*-chlorosuccinimide (120 mg, 0.63 mmol). The mixture was stirred for 2.5 h at r.t. under a nitrogen atmosphere, then 1-thio-*α*-D--glucopyranose 2,3,4,6-tetraacetate or 1-thio-*β*-D-glucopyranose 2,3,4,6-tetraacetate (0.33 g, 0.9 mmol) in DCM (5 mL) was added. The resulting mixture was treated with triethylamine (0.75 mL, 5.4 mmol). The reaction mixture was stirred for 2 h at r.t. under a nitrogen atmosphere then acidified with aqueous 1 M H_2SO_4 (7 mL/mmol of sugar). The mixture was left to stand for about 10 min and then separated. The aqueous phase was extracted with DCM (3×30 mL). The combined organic layers were dried over $MgSO_4$, filtered and the filtrate was concentrated under reduced pressure. Compound **2a** (189 mg, 40 %) was obtained as a foam by flash column chromatography on silica gel eluting with 10 % MeOH/DCM. The characterization data for **2a** are given in the Supplementary material to this paper.

Compound **2b** (150 mg, 47 %) was obtained as a foam by flash column chromatography eluting with 90 % DCM/MeOH. The characterization data for compound **2b** were identical to literature values.[10]

*Potassium salt of 1-thio-*α*- or* β*-D-glucopyranoze 2,3,4,5-tetraacetate 1-[(1Z)-5-(methylsulfinyl)-N-(sulfooxy)pentanimidate] (**3a** or **3b**)*. To a stirred solution of the thiohydroximate (**2a** or **2b**, 120 mg, 0.2 mmol) in dry pyridine (5 mL) was added pyridine–sulfur trioxide complex (95.0 mg, 0.6 mmol). After stirring at r.t. under N_2 for 24 h, an additional portion of the pyridine–sulfur trioxide complex (19.0 mg, 0.1 mmol) was added and stirring was continued for 2 h. Subsequently, a solution of $KHCO_3$ (850 mg, 8.4 mmol) in water (10 mL) was added, the mixture stirred for 30 min and then concentrated under reduced pressure. The residue was dissolved in water and extracted with chloroform (3×40 mL) and then 20 % MeOH/ /$CHCl_3$ (2×30 mL). The organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. To remove excess pyridine, the mixture was co-distilled several times with toluene. Compound **3a** was obtained as a white solid (74 mg, 50 %).

Compound **3b** was obtained by flash chromatography eluting with 80 % DCM/MeOH as a colorless solid (103 mg, 73 %). The characterization data for compound **3b** were identical to literature values.[10]

β*-Glucoraphanin potassium salt (**4b**)*. The *β*-GRP potassium salt **4b** was prepared from **3b** following literature methods and the data were identical to literature values.[10]

Spectral and analytical data of the compounds are given in Supplementary material to this paper.

Anti-inflammatory assays

The anti-inflammatory assays were conducted following the literature.[11,15] Human monocytic leukaemia THP-1 cells were obtained from the American Type Culture Collection. The cells were grown in 10 % heat-deactivated fetal bovine serum and Invitrogen RPMI-1640 containing 2 mM L-glutamine. The cytokine (TNF-*α*) ELISA kit including the reagents was obtained from BD Bioscience (R&D systems).

All compounds were dissolved in sterile distilled water then further diluted in Invitrogen DMEM (Dulbecco's modified eagle medium). The cells were grown in a 75 mL flask and

maintained at 37 °C in a humidified 5 % CO_2 atmosphere. The experiments were performed once the cells had reached 10^5 cells mL^{-1}. Phorbol 12-myristate 13-acetate (PMA) was dissolved in DMSO to a concentration of 1 mg mL^{-1} and then further diluted before use. The cells were plated out to a cell density of 10×10^4 cell mL^{-1}, at 100 µl $well^{-1}$ in a 96-well plate then treated with PMA to a final concentration of 50 nM for 24 h at 37° under a humidified 5 % CO_2 atmosphere.

Lipopolysaccharides (LPS) were dissolved in sterile water to a concentration of 5 mg mL^{-1} and then further diluted to the working stock of 10 µg mL^{-1}. The THP-1 cells were challenged with various compounds at a concentration ranging from 0.1–15 µM. They were stimulated with LPS at a final concentration of 50 ng mL^{-1}. The supernatants were collected after 4 h incubation and stored at –20 °C until enzyme-linked immunosorbent assay (ELISA) analysis.

A sandwich ELISA was used to screen the supernatants for the release of cytokine TNF-α. The ELISA plates were coated with a capture antibody (1:250) which was diluted in coating buffer and left at 4 °C overnight. The ELISA plates were aspirated and washed 3 times with 1×PBST (phosphate buffered saline with Tween-20 (0.05 % Tween-20, pH 7.4) before adding 200 µL $well^{-1}$ assay diluent and incubated at room temperature for 1 h. Standards were prepared by 2-fold serial dilutions to the range from 500–7.8 pg mL^{-1} in assay buffer diluent. Standards and sample were added in quadruplicate into appropriate wells and incubated at room temperature for 2 h. After the 2 h incubation, the plates were aspirated and washed for a total of 5 washes. The detection antibody and HRP reagent were added (100 µL $well^{-1}$) and incubated at room temperature for 1 h. The plates were aspirated and washed again, this time for a total of 7 washes and were soaked for 30 s between each wash. The substrate solutions were added (100 µL $well^{-1}$) and incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 µL $well^{-1}$ of kit stop solution then read at 450 nm with a plate reader within 30 min with a λ correction at 570 nm.

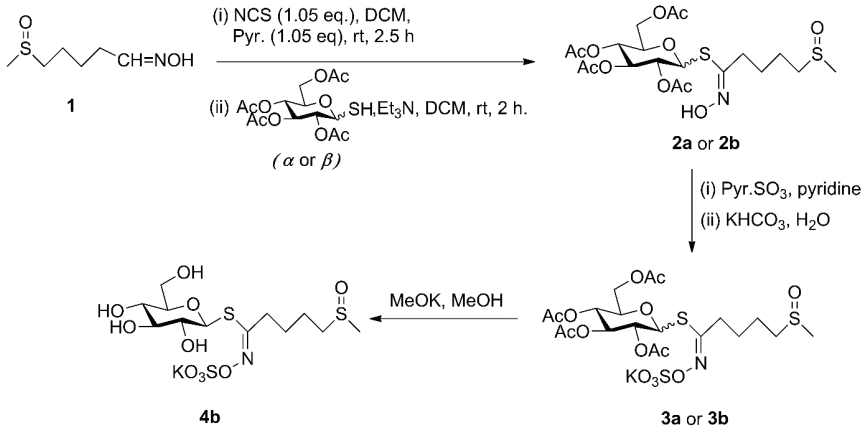
Isolation and purification of GRP from broccoli seeds

Natural GRP was isolated from broccoli seeds and purified following the literature.[16] To approximately 9 g of broccoli seeds 90 mL of boiling water was added, and the mixture boiled for 5 min. The bulk of the water was decanted and the seeds transferred to a mortar with 15 mL of water. The seeds were ground to a paste. The resultant slurry was transferred to a 200 mL volumetric flask with deionized water, made to the mark and sonicated for 5 min. The extract was filtered under vacuum through Whatman No. 4 filter paper. Mega Bond Elut C18 cartridges (3 g) were activated with methanol and washed with water. Mega Bond Elut NH_2 cartridges (3 g) were activated with methanol and equilibrated with 1 % acetic acid in water. The C18 and NH_3^+ cartridges were connected in series and 30 mL of the extract loaded onto the C18 cartridge. The cartridges were washed with 18 mL of deionized water, the C18 cartridge discarded and the NH_3^+ cartridge washed with 18 mL of methanol. The glucosinolate fraction was removed from the NH_3^+ cartridge with 30 mL of freshly prepared 2 % solution of concentrated NH_4OH solution in methanol. The solution was evaporated to dryness under a stream of nitrogen at room temperature. GRP was obtained from the crude residue by HPLC (mobile phase 1 vol. % CH_3CN, 99 % aqueous 0.1 % formic acid)[16] as a colorless liquid (35.7 mg). The NMR and MS data of the isolated GRP matched with the literature values.[10,17] The isolated GRP was then used for anti-inflammatory assays.

RESULTS AND DISCUSSION

Synthesis of α- *or* β-*glucoraphanin potassium salts*

It was shown that the oxime **1** could be synthesized from the 5-chloropentanol (Scheme 1).[10] Based on previous work, the thiohydroxymates **2a** or **2b** were formed by the coupling of oxime **1** and 1-thio-*α*-D-glucopyranose 2,3,4,6--tetraacetate[18] (or 1-thio-*β*-D-glucopyranose 2,3,4,6-tetraacetate[11]) following the Vo method.[10] Thus, oxime **1** was treated with *N*-chlorosuccinimide (NCS) in the presence of pyridine in dichloromethane (DCM) to form the hydroximoyl chloride, which was directly coupled with the *α*-thiol (or *β*-thiol) in triethylamine to yield the thiohydroxymates **2a** (or **2b**) in 47 % (or 40 %) yield in a one-pot reaction (Scheme 1).



Scheme 1. Synthesis of *α*- or *β*-GRP potassium salts.

Sulfation of **2a** (or **2b**) was accomplished with pyridine-sulfur trioxide complex in pyridine (Pyr) (Scheme 1).[19] The resulting potassium salts **3a** or **3b** (73 % and 40 % yields) were isolated by flash column chromatography on silica gel. De-*O*-acetylations were performed by dissolving **3a** (or **3b**) in MeOH in the presence of MeOK as catalyst. The final *β*-GRP potassium salt **4b** (17 % overall yield over seven steps) was successfully purified by flash column chromatography on silica gel.[10]

Unfortunately, the final *α*-epimer could not be obtained. The mass spectrogram of the reaction solution showed no peak for the $[M–K]^-$ of the *α*-GRP potassium salt, which was usually observed as the base peak ion in the spectra of *α*-GLs.[18] Attempts to achieve the *α*-isomer by using different de-*O*-acetylation conditions[20] were unsuccessful. Thus, only the potassium salt of 1-Thio-*α*- or *β*-D-glucopyranoze 2,3,4,5-tetraacetate 1-[(1*Z*)-5-(methylsulfinyl)-*N*-(sulfooxy)-pentanimidate] **3a** was obtained for the first time in 11.4 % yield over 6 steps.

Anti-inflammatory activity of the synthetic β-*GRP*

To compare the anti-inflammatory activity of the synthetic *β*-GRP (**4b**) with natural GRP and typical indole and aromatic glucosinolates,[11,21] the anti-inflammatory properties of the synthetic *β*-GRP were tested *via* an *in vitro* assay developed based on the THP-1 cell line following the literature.[11,15,21] The results are summarized in Fig. 1 and Table I.

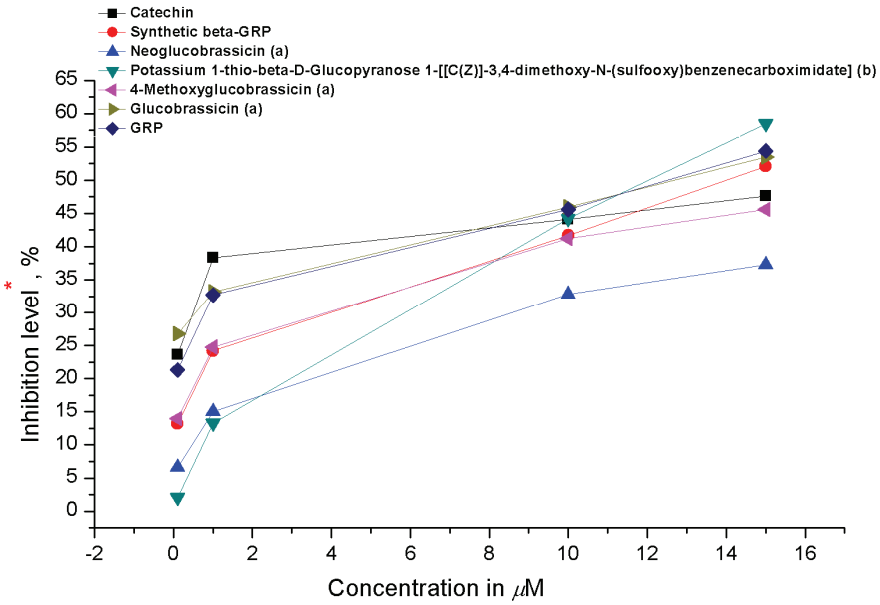


Fig. 1. Comparison of TNF-α released by LPS alone and in addition to GLs at different concentrations. LPS stimulates the release of TNF-*α*. The % inhibition is calculated from the difference between TNF-*α* released with LPS alone and in combination with the GLs (from the average of the three replicates). Moderate activity was observed for the majority of the GLs at low micromolar levels,[17] a[11], b[21].

It was shown that in the presence of synthetic *β*-GRP, TNF-*α* secretion was significantly inhibited (> 50 % inhibition) at a concentration of 15 μM, while synthetic *β*-GRP exhibited higher inhibition than neoglucobrassicin at all testing concentrations, reaching nearly the same point as glucobrassicin but higher than 4-methoxyglucobrassicin at a concentration of 15 μM (> 50 %, Fig. 1).[11] Comparison with the positive control (catechin) showed that at concentrations lower than 10 μM, most of the synthetic GLs showed lower inhibition than catechin, but at a concentration of 15 μM, *β*-GRP exhibited higher activity than catechin (52 % inhibition for *β*-GRP compared with 48 % inhibition for catechin). The trend was almost opposite in comparison with potassium 1-thio-*β*-D-glucopyranose 1-[[*C*(*Z*)]-3,4-dimethoxy-*N*-(sulfooxy)benzenecarboximidate] (the glucosin-

olate with the highest anti-inflammatory activity in the aromatic glucosinolate family[18]). The results showed that synthetic *β*-GRP reached nearly the same inhibition of natural GRP at a concentration of 15 μM (52 % inhibition by *β*-GRP, 54 % inhibition by natural GRP); the trends were similar at higher investigated concentrations. Thus, it was clearly demonstrated that the synthetic *β*-GRP has significant anti-inflammatory activity at low concentrations (≈50 % inhibition at a concentration of 15 μM). The results of the biological activity of synthetic GRP, consistent with previous studies,[8,9] provide ample evidence for the potential of synthetic GRP for medicinal uses.

TABLE I. Effects of synthetic GLs on TNF-*α* secretion in LPS-stimulated THP-1 cells

Treatment	Content of TNF-*α* secretion (*SD*)[a], pg mg^{-1}
LPS (50 μg L^{-1})	488.68 (15.81)[b]
LPS + 15.00 μM catechin	255.94 (33.77)
LPS + 10.00 μM catechin	273.02 (21.53)
LPS + 1.00 μM catechin	301.41 (20.14)
LPS + 0.10 μM catechin	373.30 (25.14)
LPS (50 μg L^{-1})	236.16 (65.15)[b]
LPS + 15.00 μM β-GRP	113.07 (39.42)
LPS + 10.00 μM β-GRP	137.63 (52.90)
LPS + 1.00 μM β-GRP	178.98 (47.48)
LPS + 0.10 μM β-GRP	204.98 (52.34)
LPS (50 μg L^{-1})	253.15 (45.53)[b]
LPS + 15.00 μM GRP	115.38 (14.45)
LPS + 10.00 μM GRP	137.69 (32.82)
LPS + 1.00 μM GRP	170.26 (41.76)
LPS + 0.10 μM GRP	199.33 (29.51)

[a]The results are for 3 different experiments run in duplicate; [b]$p \leq 0.07$, compared with control

CONCLUSIONS

The total synthesis of *α*- or *β*-GRP (potassium salts) was attempted. While the *β*-epimer was successfully synthesized in high overall yield (17 % over seven steps), *α*-GRP was found to be unstable as it decomposed in the final step. By observing the inhibition of the release of TNF-α in LPS-stimulated THP-1 cells, it was shown that the synthetic *β*-GRP and natural GRP have similar, significant anti-inflammatory activity at low concentrations. The obtained results raise the possibility of developing a GRP-based nutraceutical for therapeutic and/or preventive medicine purposes.

SUPPLEMENTARY MATERIAL

Analytical and spectral data of the compounds are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

АНТИ-ИНФЛАМАТОРНА АКТИВНОСТ СИНТЕТИЧКОГ И ПРИРОДНОГ ГЛУКОРАФАНИНА

QUAN V. VO[1,2], PHAM C. NAM[3], THUC N. DINH[4], ADAM MECHLER[5] и THI T. V. TRAN[6]

[1]Department for Management of Science and Technology Development, Ton Duc Thang University, Ho Chi Minh City, Vietnam, [2]Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam, [3]Department of Chemical Engineering, University of Da Nang – University of Science and Technology, Vietnam, [4]Faculty of Natural Sciences, Hong Duc University, Thanh Hoa, Vietnam, [5]La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia и [6]Department of Chemistry, Hue University of Science, 77 Nguyen Hue, Hue, Vietnam

Глукорафанин је због својих корисних лековитих карактеристика један од најпознатијих глукозинолата. Познато је да деловањем једињења долази до уклањања канцерогена у ткивима, и услед тога су често изучаване превентивна антиканцерска својства једињења. Успешно је синтетисана калијумова со β-глукорафанина, у високом укупном приносу, док је утврђено да је α-епимер нестабилан и да се разграђује у финалном кораку синтезе. Антиинфламаторна активност глукорафанина је испитана одређивањем инхибиције ослобађања фактора некрозе тумора-алфа (TNF-α) из THP-1 ћелија стимулисаних липополисахаридима. Показано је да је у присуству синтетичког или природног глукорафанина, при концентрацијама 15 μM значајно инхибирана (≈ 52 % инхибиције) секреција TNF-α.

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