Antioxidant and Anti-inflammatory activity of PEG-Indol-3-Carbinol conjugates

Alessia Fazio[a], Domenico Terenzio[b], Anna Lisa Piccinelli[b] and Luca Rastrelli[b]

[a]Department of Pharmacy, Health and Nutritional Science, University of Calabria, 87036 Arcavacata di Rende (CS), Italy

[b]Department of Pharmacy, University of Salerno, 84084 Fisciano (SA), Italy

Abstract

I3C is a very active molecule but it presents limits that often prevent their success. Most common problems are low half-life, due to rapid kidney clearance and to rapid inactivation by metabolic enzymes, instability in water and pH gastric, low molecular weight, low water solubility (7 mg/ml, insoluble in cold water) and low selectivity towards cancer cells.

In this paper a series of PEG–Indol-3-carbinol conjugates were synthesized in order to overcome some of the limits of I3C, especially the low molecular weight ones. In particular, some polymeric conjugates were prepared by linking I3C to a PEG_{550}-COOH and HOOC-PEG_{600}-COOH polymer that acts like a carrier. In fact, polymeric conjugation allows to improve drug pharmacokinetic profiles by reducing drug clearance, and protects the drug from enzymatic degradation and can target the linked I3C to tumor tissues. The total antioxidant activity of I3C conjugates was determined by the ABTS and chemiluminescence assays and compared with the values of other antioxidant compounds. *In vitro* assays showed that the I3C-PEG conjugates have significant anti-inflammatory effects, inhibiting NO release in the LPS-induced J774.A1 murine macrophage cell line.

Introduction

The human diet offers a greater and more varied group of plant bioactive compounds than do drugs, but people often do not realize that many drugs are derived from the metabolites originally discovered in food plants and vegetables.

Numerous epidemiological studies indicate that dietary consumption of Brassica (or cruciferous) vegetables, such as Brussel's sprouts, mustard greens, radish, rutabaga, turnip, cabbage, cauliflower and broccoli, provides protection against some chronic diseases and specific cancers [1], since they are rich sources of glucosinolate derivatives. Indole glucosinolates are a group of sulphur-containing secondary metabolites derived from tryptophan which are biologically active. Indole-3-carbinol (I3C) and 3,3-diindolylmethane (DIM) are naturally occurring plant alkaloids formed by the hydrolysis of indolylmethyl glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli and brussels sprouts [2]. The amount of I3C found in the diet can vary greatly, ranging from 20 to 120 mg daily, and is dependent on dietary intake of cruciferous vegetables and their changeable concentrations [3].

When the plant cells are damaged as by cutting or chewing, a thioglucosidase-mediated autolytic process takes place generating I3C, glucose, and thiocyanate ion. Studies have shown a correlation between diets high in cruciferous vegetables and the reduced incidence of several types of cancer. I3C has been proposed as one of the causative agents for these observations. The known estrogenic activity of I3C lends some support to this idea. I3C provides powerful antioxidant protection, helping to prevent cellular damage caused by free radicals. In addition, I3C can help to maintain healthy normal hormonal balance, for both men and women and therefore may support the health of the breast, prostate, and other reproductive organs. I3C is also known to support the liver's detoxification and act as antiplatelet and anti-inflammatory agent. Due to the extensive scientific research that has been generated on its safety and efficacy, a lot of company have chosen to provide I3C in supplement form.

Indole-3-carbinol works as a strong antioxidant, thereby protecting the DNA and other cell structures. I3C and its metabolites at the concentration range of 50–200 M have suppressive effects on the proliferation of various cancer cell lines including those of breast, colon, prostate, and endometrium by targeting a wide spectrum of signaling pathways governing hormonal homeostasis and inhibits spontaneous or chemical-induced tumorigenesis in mammary gland, liver, cervix and gastrointestinal tract in different animal model studies. It is reported that I3C inhibits the migration and invasion of the breast cancer cells and reverses the multiple drug resistant phenotype of human leukemia cells and murine melanoma cells [4].

I3C Studies shown that I3C treatment can arrest the PC-3 prostate cancer cells in G1/G2 phase. They analyzed that p21 and p27 and their inhibitory association with cyclin D1 and cyclin E are up regulated with using I3C. Also down-regulation of CDK6 protein kinase levels and activity and Rb phosphorylation is associated with them [5]. Besides them amount of active p53 and its binding the p21 promoter is increased by I3C treatment. And when p53 amount was reduced using small interfering RNA, it is prevented the cell cycle arrest in G1/G0 phase. Also I3C induce to upregulates p21 when inducing G1/G0 cell cycle arrest in HCT 116 colon cancer cells [6].

In many studies was assessed the *in vitro* and *in vivo* antiplatelet and antithrombotic effects of I3C [7]; moreover I3C has recently received attention as a possible anti-obesity agent, useful in metabolic syndrome and related disorders. I3C ameliorates adipogenesis by activating SIRT1 in 3T3-L1 cells [8]. I3C positively regulate the carbohydrate metabolism enzymes in high-fat diet mice showing anti-hyperglycemic action comparable with the drug metformin [9]. However, several factors compromise the *in vivo* efficacy of

indole-3-carbinol, including low *in vitro* antitumor potency, limited bioavailability, and complicated pharmacokinetic behaviors due to intrinsic metabolic instability [4]. Recent studies by Bradlow et al. demonstrated that I3C is unstable in cell culture medium and physiological fluids [10]. More than 50% of I3C converted to 3,3-diindolylmethane (DIM) within 24 h. Additionally, when taken orally and exposed to the acidic environment of the stomach, I3C is readily converted to several condensation products including DIM, 2-(indol-3-ylmethyl)-3,3-diindolylmethane (LTr1), indolylcarbazole (ICZ),and 1-(3-hdroxymethyl)-indolyl-3-indolylmethane (H1-1M) [11]. Consequently, the structural optimization of indole-3- carbinol or its metabolite 3,3'-diindoylmethane (DIM) to develop novel indole derivatives with improved potency and metabolic stability has been the focus of many recent investigations.

In this paper we have prepared stable conjugates by covalently linking indole-3-carbinol (I3C) to poly(ethylene glycol) (PEG) that was chosen as carrier thanks to its interesting features: (i) lack of immunogenicity, antigenicity and toxicity; (ii) high solubility in water and in many organic solvents; (iii) high hydration and flexibility of the chain, which is at the basis of the protein rejection properties; and (iv) FDA approval for systemic human use. PEG is synthesized by ring opening polymerization of ethylene oxide using methanol or water as initiator to yield methoxy–PEG or diol PEG, respectively. It has been used for a long time as excipient in many pharmaceutical or cosmetic formulations [12] while studies in the field of drug conjugation and delivery started only 30 years ago. I3C -PEG coniugates were subjected to ABTS and chemiluminescence tests in order to determine their antioxidant capacity and the results obtained compared with those of I3C and other antioxidant compounds used as reference. In addition, the anti-inflammatory properties were evaluated *in vitro* using lipopolysaccharide (LPS)-stimulated J774.A1 murine macrophage by investigating the production of nitric oxide.

I3C -PEG coniugates could meet the following advantages: increase of I3C half-life by reducing its kidney clearance, protection of I3C from enzymatic degradation, enhance of I3C water solubility.

Experimental

Chemicals and reagents. Poly(ethylene glycol) methyl ether (average Mn 550), poly(ethylene glycol) bis (carboxymethyl) ether (average Mn 600), potassium *ter*-butoxide (≥ 98%), *tert*-butanol anhydrous (≥ 99.5%), *tert*-butylbromo acetate (98%), trifluoroacetic acid (99%), N-hydroxysuccinimide (98%), *N,N*'-dicyclohexylcarbodiimide (≥ 99%), indole-3-carbinol and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were purchased from the Sigma Chemical Co. (Milan, Italy). Dichloromethane, toluene, diethyl ether and triethylamine were also obtained from Sigma Aldrich. Deionized water was prepared using a Milli-Q reverse osmosis unit from Millipore (Bedford, MA, USA).

LPS was obtained from Sigma–Aldrich (Schnel-ldorf, Germany). Griess reagents and nitrite standard were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Synthesis of PEG$_{550}$-O-CH2-COOH (1)

Linear mPEG-OH (average Mn 550) was modified to mPEG-COOH as previously reported [13].

Briefly, for synthesis of PEG$_{550}$-O-CH$_2$-COOH, poly(ethylene glycol) methyl ether (average Mn 550, 1.106 mg, 2 mmol) was added to 30 ml of toluene and dehydrated by Dean Stark distillation. To the remaining PEG solution (5 mL), anhydrous *tert*-butanol (10 mL) and potassium *ter*-butoxide (224.42 mg, 2 mmol) were added and the solution was left at 50°C for 1 h, under stirring. *Tert*-butylbromo acetate, (295.2 μL, 2mmol), were added and the mixture, heated at reflux for 1h, and the reaction was allowed to proceed for 18 h at room temperature. KBr was removed by Celite

filtration and the solvent evaporated under vacuum to give mPEG-O-CH$_2$-COOtBu (yield: 1.33 g, 98%). The ester was hydrolyzed by treating with 15 mL of TFA, 20 mL of CH$_2$Cl$_2$ and 3 μL of H$_2$O for 3h. The reaction mixture was concentrated under vacuum and the TFA was removed under reduced pressure to give mPEG$_{550}$-O-CH$_2$-COOH (yield: 1.12 g, 92%). The identity of the product was confirmed by thin-layer chromatography (TLC), (mobile phase: CHCl$_3$/methanol at 90:10).

see Scheme 1.

Synthesis of PEG$_{550}$-O-CH$_2$-COO-NHS (2)

PEG monomethyl ether with one end of carboxyl group was first activated with NHS. Briefly, PEG$_{550}$-O-CH$_2$-COOH (0.900 g, 1.48 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (60 ml). To the solution N-hydroxysuccinimide (NHS) (204.4 mg, 1.77mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (366.,44 mg, 1.77mmol) were added. The mixture was stirred for 5 h at room temperature. After filtration of the N,N'-dicyclohexylurea, solution was added dropwise into 250 ml of diethyl ether to precipitate excess NHS, followed by centrifugation and filtration. The solvent was removed to afford PEG$_{550}$-O-CH$_2$-COO-NHS (yield: 0.987 g, 94.6%).

see Scheme 2.

Synthesis of PEG$_{550}$-INDOLE-3-CARBINOL (3)

Indole-3-carbinol (273.75 mg, 1.86 mmol) and Et$_3$N (258,53 μL, 1.86 mmol) were dissolved in anhydrous CH$_2$Cl$_2$ (50 mL). Subsequently, PEG$_{550}$-O-CH$_2$COO-NHS (0,987 g, 1.4 mmol) was slowly added to the reaction mixture over a period of 30 min and the mixture was stirred at room temperature for 48 h. The progression of the reaction was monitored by TLC. The solvent and Et$_3$N were removed under vacuum to afford mPEG$_{550}$-indole-3-carbinol.

see Scheme 3.

Synthesis of NHS-OOC-PEG$_{600}$-COO-NHS (4)

The same chemical strategy was followed for the synthesis of the dual functional PEG. Poly(ethylene glycol) bis (carboxymethyl) ether (average Mn 600, 1.200 g, 2 mmol) was dissolved in 30 ml of toluene and dehydrated by Dean Stark distillation. To the remaining PEG solution (5 ml) dissolved in anhydrous CH$_2$Cl$_2$ (80 mL), N-hydroxysuccinimide (NHS) (552.432 mg, 4.8 mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (990.384 mg, 4.8 mmol) were added. The mixture was stirred for 18 h at room temperature. After filtration of N,N'-dicyclohexylurea (DCU) the solvent was removed under vacuum to give the product (yield: 1.16 g, 73%).

see Scheme 4.

Synthesis of INDOLE-3-CARBINOL-PEG$_{600}$-INDOLE-3-CARBINOL (5)

Indole-3-carbinol (706.464 mg, 4.8 mmol) and Et$_3$N dry (667,2 μL, 4.8 mmol) were dissolved in anhydrous CH$_2$Cl$_2$ (70 mL). Subsequently, NHS-OOC-PEG$_{600}$-COO-NHS (1 g, 2 mmol) was slowly added and to the reaction mixture over a period of 30 min and the mixture was stirred at room temperature for 48 h. The progression of the reaction was monitored by TLC (thin layer chromatography). The solvent and Et3N were removed under vacuum to afford indole-3-carbinol-PEG$_{600}$-indole-3-carbinol.

see Scheme 5.

ABTS Radical Cation Decolorization Assay. Evaluation of free radical scavenging activity was performed with the Trolox equivalent antioxidant activity (TEAC) assay. TEAC value is based on the ability of the antioxidant to scavenge the preformed radical of 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+), with spectrophotometric analysis, according to the method of Re et al. [14]. Samples were diluted with MeOH to have 0.3, 0.5, 1.0, 1.5, and 2.0 mM solutions. The reaction was enhanced by the ad-

diction of 1.0 mL of diluted ABTS to 10 μL of each solution of sample or Trolox (standard) or 10 mL of MeOH (control). The determination was repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration as a function of the control's absorbance, 1 min after initial mixing. The antioxidant activity was expressed as TEAC (Trolox equivalent antioxidant activity), which is the concentration of standard Trolox solution with equivalent percentage inhibition to a 1 mM solution of the tested compounds.

Chemiluminescence Assay. Total antioxidant capacity was assayed by chemiluminescence according to Whitehead et al.[15]. Enhanced chemiluminescent signal reagent (Amersham, UK) comprising assay buffer and tablets A and B (containing luminol, *p*-iodophenol enhancer and perborate oxidant), was prepared by adding tablets A and B to the buffer solution. Signal reagent (0.4 mL) was added to distilled water (1 mL) in a glass cuvette containing a magnetic stirrer. The cuvette was placed in a Perkin Elmer Wallac Victor 2 Chemiluminometer and the reaction commenced by addition of 25 μL horseradish peroxidase (4 μg mL^{-1} in H$_2$O). Compounds (100 μL of 0.5 mg/mL dissolved in PBS, pH 7.4) were added to the cuvette and the time for which light output was suppressed was determined. Comparison was made with a standard curve generated using Trolox (20 mg mL^{-1} in H$_2$O).

Analysis of Nitrite. J774A.1 cells (5.0 × 104 cells/well) were plated on 96-well microtiter plates and allowed to adhere at 37 °C in a 5% CO$_2$ atmosphere for 2 h. Examined extracts and compounds (1-100 mg/mL for the extracts and 1-100 μg/mL for compounds) were added 1 h before and simultaneously to LPS (6 × 103 U/mL), used to induce inducible iNOS. Nitric oxide release, evaluated as nitrite (NO2$^-$) accumulation in the cell culture medium, was performed 24 h after LPS stimulation by the Griess reagent [16]. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results are expressed as percentages of inhibition calculated versus cells treated with LPS alone. *N*(G)-Nitro- L-arginine methyl ester (L-NAME; 1 μM) has been used as reference drug, able to inhibit nitrite production by LPS-treated macrophages, giving rise to 46.56 (1.25% inhibition of nitrite release versus LPS alone (data not shown in Table 2).

Results

We have prepared stable I3C-PEG conjugates in order to improve the metabolic instability of indole-3-carbinol. The synthesis was realized by PEGylation process in which I3C was covalently linked to mPEG-COOH$_{550}$ and HOOC-PEG$_{600}$-COOH. Poly(ethylene glycol) (PEG) was chosen as polymeric carrier thanks to its interesting features, in particular biocompatibility, highly solubility and FDA approval for systemic human use.

PEG monomethyl ether with one end of indole-3-carbinol (mPEG-I3C, Mw = 550) and a dual functional PEG with two ends of indole-3-carbinol (I3C-PEG-I3C, Mw =600) were synthesized. First, linear mPEG-OH was modified to mPEG-OCH2COOH (1, **Scheme 1**). Subsequently, free carboxylic groups of PEG- OCH$_2$COOH and HO-OC-PEG-COOH were activated by NHS/DCC [eqn (1) and (3)] as previously reported [13] with some modification.

The activation was achieved using a molar ratio –COOH:NHS: DCC equal to 1:1.2:1.2, less than the amounts previously used (6 moles of NHS and DCC per mole of carboxyl group). The use of only a slight excess of NHS and DCC (0.2 instead of 5) was needed to avoid problems in the subsequent purification of the product, since it was impossible to separate for precipitation the activated PEGs from excess unreacted reagents of low molecular weight, because of the low molecular weight average of starting PEGs. Ne-

vertheless PEG_{550}-O-CH_2-COO-NHS (2) and NHS-OOC-PEG_{600}-COO-NHS (4) were obtained in high yields (94.6% e 73%, respectively). Furthermore, the activation reaction was carried out using a smaller volume of solvent (mmol of the starting PEG per mL of CH_2Cl_2 was equal to 0.025 instead of 0.015). PEGylated I3C, PEG_{550}-indole-3-carbinol (3) and indole-3-carbinol-PEG_{600}-indole-3-carbinol (5) were synthesized through the reaction of the activated carboxylic groups of PEGS with the hydroxyl end of indole-3-carbinol [eqn (2) and (4)] leading to the formation of an ester bond.

The free-radical scavenging activity PEG-I3C conjugates was evaluated using ABTSand and chemiluminescence (CL) assays. The results obtained were compared with the values of antioxidant capacity of flavonoids, cinnammic acids and vitamins and also with the antioxidany activity of I3C.

As shown in Table 1, our polymeric conjugates showed antioxidant activity higher than that of the I3C and other compounds. These data confirmed the role played by the I3C derivatives as antioxidants. From all data reported herein we can state that PEG-I3C may improve antioxidant status and possibly reduce risk of chronic disease associated with oxidative stress. Further studies are necessary to confirm if the evident potential of this new derivatives offers a real and useful in vivo improvement of the antioxidant status.

see Table 1.

The pharmacological activities of I3C have been extensively reviewed. In particular it has been shown to possess anti-inflammatory activity [17] which led us to investigate the in vitro activities of our I3C conjugates PEG_{550}-I3C and I3C-PEG_{600}-I3C on inducible nitric oxide synthase (iNOS) activity, evaluating nitrite production, index of nitric oxide (NO) biosynthesis, in the medium of LPS activated J744.A1 macrophage. NO release in the cellular medium of LPS-stimulated J774.A1 macrophages, incubated with I3C, PEG_{550}-I3C and I3C-PEG_{600}-I3C (1, 10 and 100 μg/mL) were evaluated 24 h after LPS (6×10^3 u/mL) challenge. Results were expressed as % of inhibition evaluated versus macrophages treated with LPS alone. All compounds, added 1 h before and simultaneously with LPS, significantly inhibited in a concentration-related manner nitrite release (P<0.001 vs LPS) for the concentrations of 10 and 100 μg/mL.

see Table 2.

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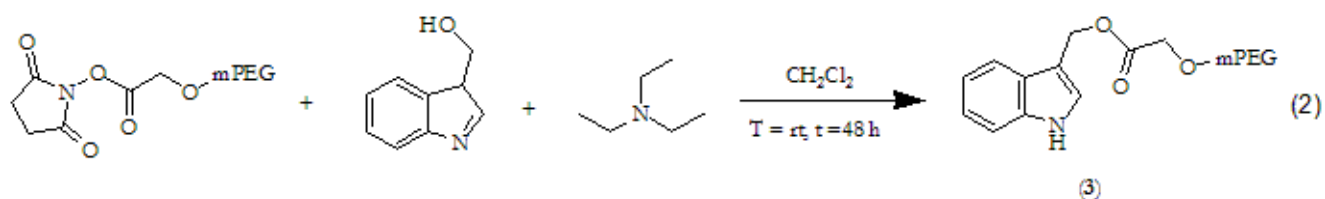
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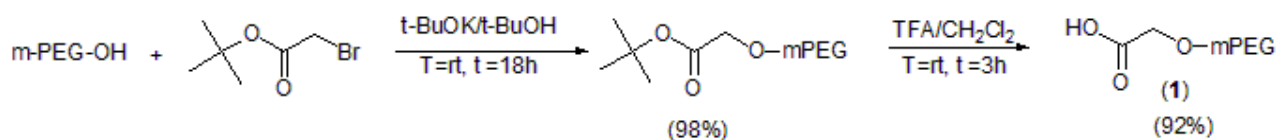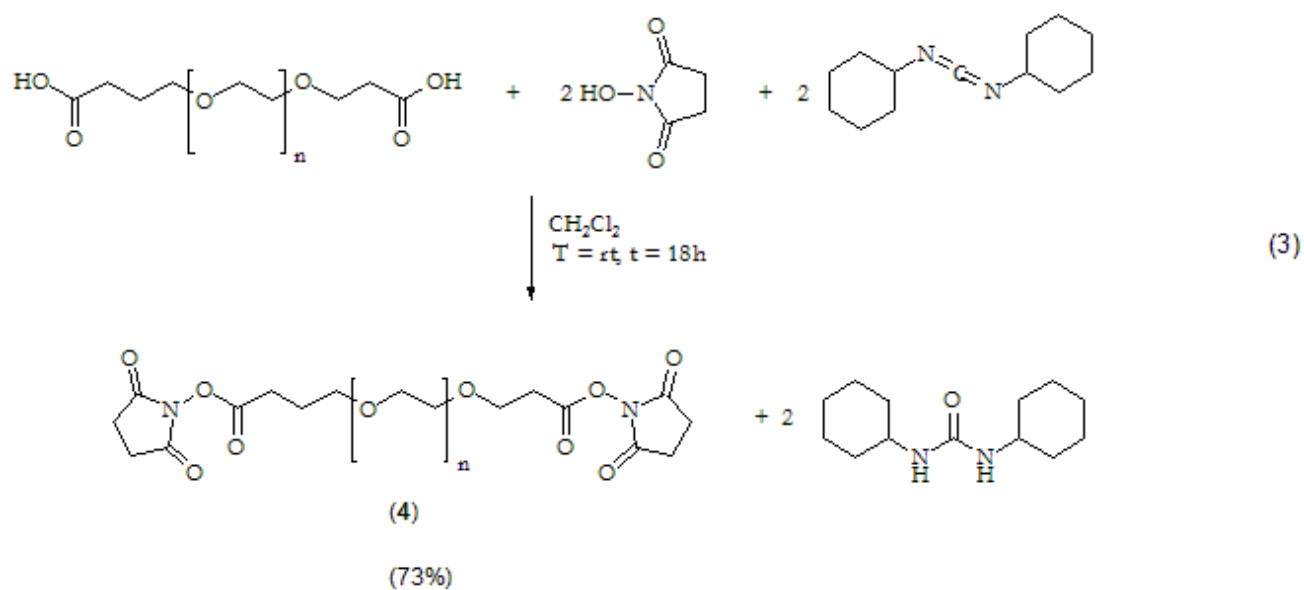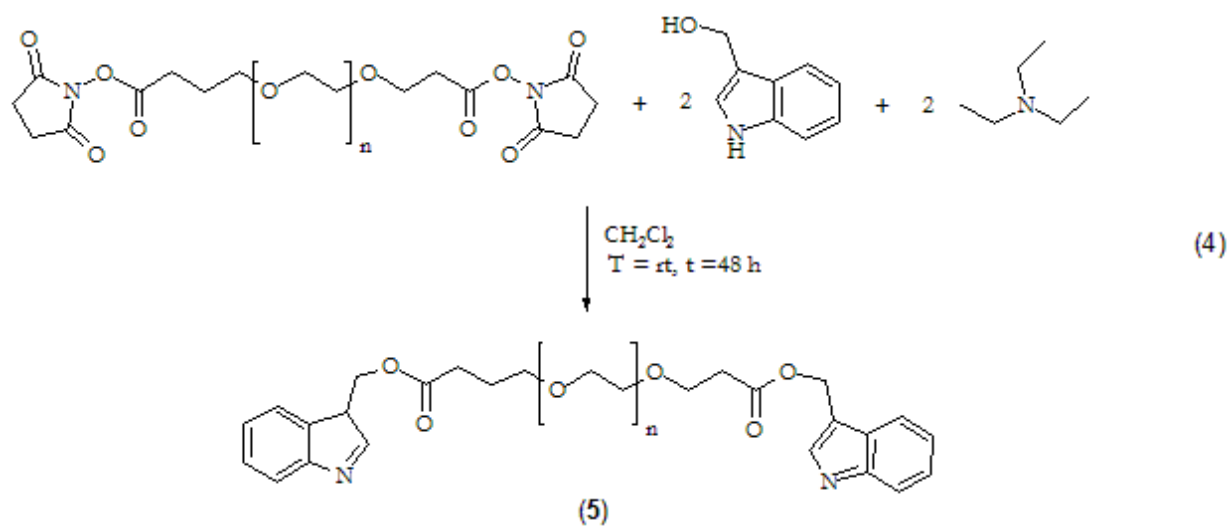
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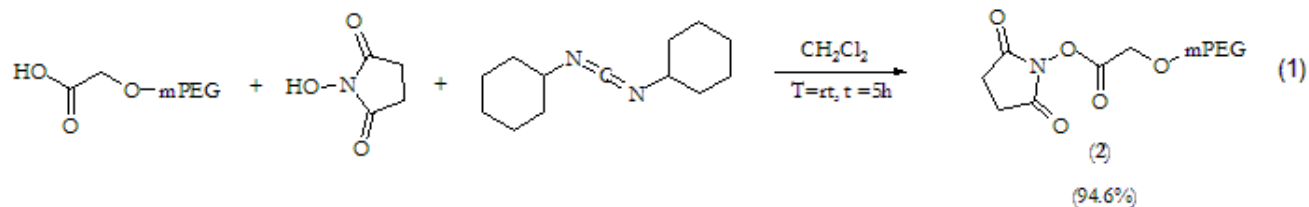
Scheme 2

Scheme 3

Scheme 4

Scheme 5

Table 1. Antioxidant activity of I3C Conjugates in the TEAC and CL Assay [a]

Compounds	TEAC assay (mM) ± SD[b]	CL assay (mM) ± SD[b]
I3C	3.8 ± 0.12	3.2 ± 0.11
PEG$_{550}$-I3C	4.2 ± 0.26	4.1 ± 0.17
I3C-PEG$_{600}$-I3C	5.8 ± 0.41	4.7 ± 0.32
Verbascoside	4.9 ± 0.22	3.6 ± 0.21
Rutin	2.1 ± 0.09	2.5 ± 0.11
Caffeic acid	5.2 ± 0.12	4.8 ± 0.10
Clorogenic acid	4.3 ± 0.13	3.4 ± 0.14

[a] For protocols used, see Experimental Section.

[b] n = 3. Results are expressed in terms of mM Trolox equivalent.

Table 2. Effect of I3C and I3C conjugates (PEG$_{550}$-I3C and I3C-PEG$_{600}$-I3C) on Nitrite Release by J774.A1 Macrophages Stimulated With *E. coli* Lipopolysaccharide (LPS)[a]

Compound	Nitrite release inhibition (% vs LPS)		
	100 µg/mL	10 µg/mL	1 µg/mL
I3C	83.1 ± 1.2***	67.0 ± 0.9***	32.0 ± 1.4
PEG550-I3C	82.5 ± 2.4***	66.1 ± 1.2***	27.5 ± 0.8
I3C-PEG600-I3C	92.3 ± 3.8***	74.7 ± 1.9****	41.1±1,4***

Data are expressed as inhibition percentage means ± s.e.m. vs nitrite production in 24 h by J774.A1 macrophages treated with LPS alone. *** and denotes P<0.001 *vs LPS treated* macrophages.