doi.org/10.36721/PJPS.2021.34.5.SP.2003-2008.1

Natural products embedded crown ethers as potent insulin secretory agents

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Abstract: Natural products embedded crown ethers were prepared by utilizing bioactive natural products including chrysin, tetrahydroisoquinoline (THIQ), and biochanin-A. The prepared crown ether scaffolds were evaluated and compared with their natural product precursors for insulin secretory activity on isolated mice islets and for their fluorescent properties. All the crown adducts were found more active as compared to their natural product precursors. Bischrysin 32-crown-10 (6d), THIQ 15-Crown-5 (6a) and chrysin 16-crown-5 (6c) showed mild, moderate and strong insulin secretory activity, respectively when compared with the standard drug tolbutamide (TB). Particularly crown derivative 6c showed strong activity (31.10 ng/islet/h) that is almost two (02) fold higher than that of standard drug TB (16.82 ng/islet/h). To the best of our knowledge crown ethers based antidiabetic study is being reported for the first time in literature through this work. Furthermore, fluorescence study showed the significant increase in absorption and emission maximum (hypsochromic effect) in crown structures when compared with their natural product precursors. Present optimistic results obtained from this study may be a guided template for developing new effective insulin secretory agents.

Keywords: Natural products, chrysin, crown ethers, insulin secretory activity, fluorescent probes.

INTRODUCTION

Diabetes mellitus (types 1 and 2) is a serious illness which may cause disability and mortality (Bais *et al*., 2017). It is caused by the insufficient or ineffective production of insulin by β-cells of pancreas which triggers inadequate concentrations of glucose in blood (Bais *et al*., 2017). In type 2 diabetes, mainly exist in Asia, deficiency in insulin secretion is more dominant than insulin resistance (Kyoto declaration, 2013). Large numbers of chemical agents are being used to control diabetes, however, they suffer harsh side effects and associate with limited recovery as well (Mishra *et al*., 2020). Alternatively, natural products proved more successful to treat diabetes *in vitro*, *in vivo* and in clinical studies as being more affordable, effective and with fewer side-effects (Sahil *et al*., 2019). As a result, pharmaceutical investigations are diverting increasingly towards the discovery of new antidiabetic agents from natural products (Alam *et al*., 2018).

Natural motifs and their derivatives indeed offer an important role to regulate living systems (Alam *et al*., 2018; Majhi and Das, 2021), consequently their complexation in crown scaffold has already gained considerable biomedical interest (Schneider *et al*., 2013). Similarly crown ethers, for example, 1-3 (fig. 1) (Ihara *et al*., 1997; Marjanović *et al*., 2007; Liu *et al*., 2008) have demonstrated their significance in drugs design owing to their ability to form non-covalent interactions with biopolymers which results in the regulation of enzyme activity (Li and Loh, 2008).

In our continuing interest to explore bioactive molecules (Arshad *et al*., 2018; Ullah *et al*., 2021) and encouraged by the notable co-relation of crown ether motifs with enzymes (Li and Loh, 2008), we aimed to synthesize natural product-embedded crown ethers by incorporating the natural molecules and the crown ether chain to investigate its potential as antidiabetic agents. To the best of our knowledge crown ether based antidiabetic study is being reported for the first time through this work. Natural compounds like tetrahydroisoquinoline (THIQ), chrysin (5,7-dihydroxyflavone) and biochanin–A, are already demonstrated a vast spectrum of pharmaceutical activities (Faheem *et al*., 2021; Stompor-Gorący *et al*., 2021; Sundaresan *et al*., 2018), to this reason, we became interested to make crown ethers of these natural skeletons. Furthermore, it was also appealing to screen these novel crown ethers for their fluorescent potential considering the already demonstrated applications of the crown ether moiety as fluorescent probes. These fluorescent probes are used to monitor the biological process *in vivo* as a potential diagnostic tool and for bio-imaging (Li *et al*., 2017).

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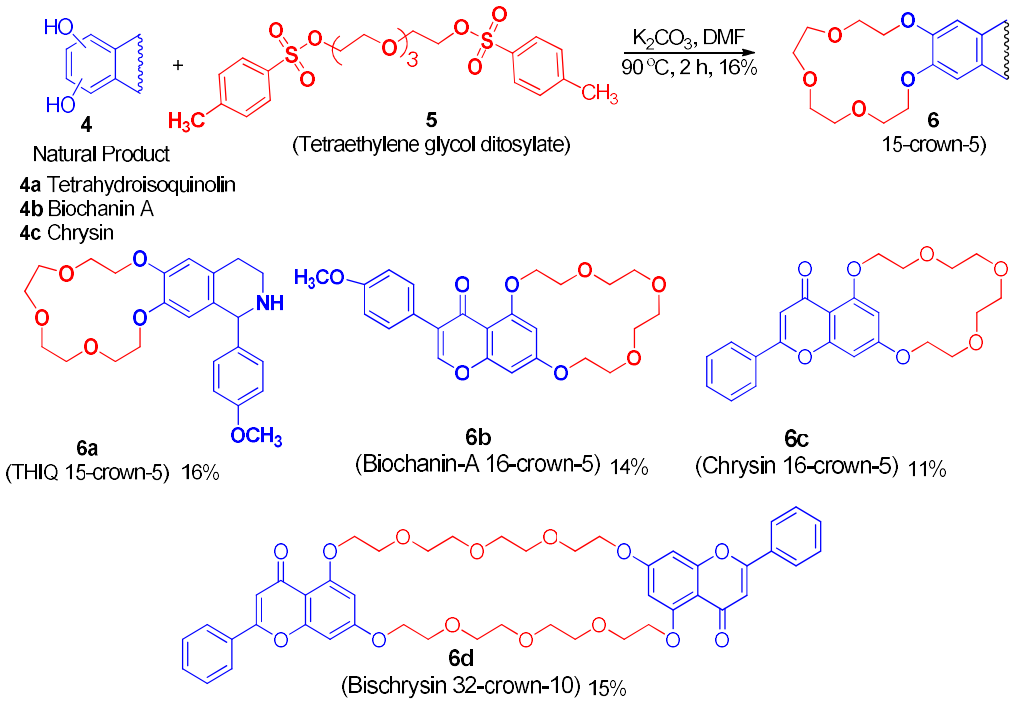


Scheme 1: Synthesis of natural products embedded crown ethers. Reagents and conditions: i) natural products 4a-c (0.3 mmol), powdered K_2CO_3 (1.2 mmol, 4 equiv.) and anhydrous DMF (1.5 mL) 90 ºC, 1 hour; then tetraethylene glycol ditosylate 5 (0.36 mmol, 1.2 equiv.) dropwise addition through septa in 1 hour; then 90 ºC, 2 hours.

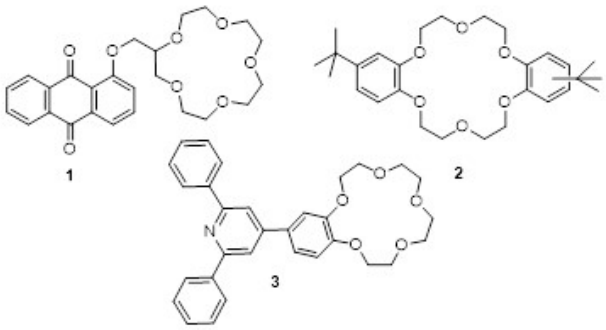


Fig. 1: Important Crown ethers with biological and fluorescent properties.

MATERIALS AND METHODS

Materials used in synthetic protocol

Potassium carbonate (K_2CO_3), dimethylformamide (DMF), tetraethylene glycol ditosylate, sephadex (LH-20, 18-111 µm) and pre-coated silica gel 60 HF_{254} plates.

General experimental details

Bruker spectrometer (AV-600) was used for recording NMR spectra. The required adducts were made pure on Sephadex column and then by using preparative thin layer chromatography (TLC) on silica plates. All reagents were purchased commercially from Sigma Aldrich. Melting point apparatus Buchi-545 was used for the determination of melting points.

General procedure for the synthesis of natural products embedded crow ethers (6a-d)

0.3 mmol of corresponding natural precursor (4a-c), 1.2 mmol of K_2CO_3 (powdered) with 1.5 mL DMF were taken in a 50 mL round bottom flask, closed with septa. The mixture was allowed 10 minutes stirring with N_2 purging then oil bath heating at 90 ºC was attained slowly. Thereafter, 1.8 mmol of tetraethylene glycol ditosylate (5) was added dropwise through septa in 1 hour duration. Reaction completion was observed in 2 hours through TLC monitoring. Reaction mixture was dried under high vacuum then purified by Sephadex column. Further purification of obtained fractions was achieved by preparative TLC to deliver pure crown adducts 6a-6d. Characterization data of crown products (6a-d) was found very similar to previous report (Iqbal *et al*., 2021).

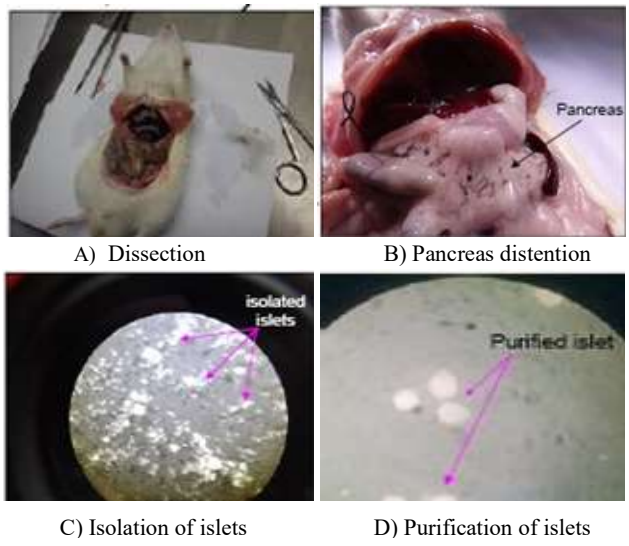
Materials for Biological Assay Protocol

Collagenase V, Tolbutamide (TB), Krebs-Ringer Modified Buffer (KRB) and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Mouse ultra-sensitive insulin ELISA kit was acquired from Crystal Chem Incorporation (IL, USA).

Animals and ethics

For this study, male BALB/c mice (28-35g) were acquired from the animal house of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. Animal studies were carried out after getting permission (Animal study protocol number:

2018-0008) from the Institutional Ethics Committee, University of Karachi, Karachi-75270, Pakistan.



A) Dissection B) Pancreas distention

C) Isolation of islets D) Purification of islets

Fig. 2: Islet isolation from mice pancreas

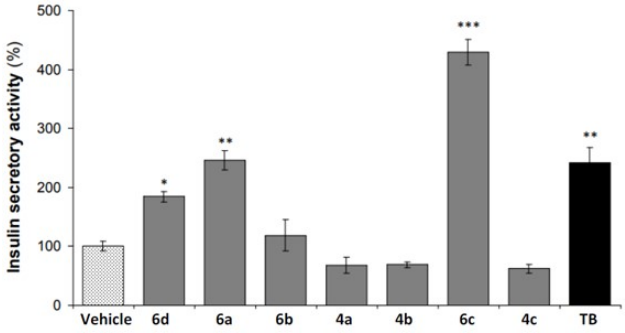


Fig. 3: Insulin secretory activity of test compounds (For detailed conditions: see table 1 footnote). Insulin secretory activity by 17 mM glucose alone (vehicle) was expressed as 100%. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. vehicle. Standard drug TB (tolbutamide, 200 µM).

Islets Isolation and insulin secretory assay protocol

Islets were isolated from BALB/c mice (28-35g) pancreas by collagenase digestion method as described in literature (Sharma *et al*., 2015). Briefly, followed by islets isolation, incubation of three (03) medium sized islets in groups was performed in glucose (17 mM) with or without test compound (200 µM) or tolbutamide (200 µM) for the duration of 60 minutes. Subsequently, supernatant (100 µl) was used to measure the secreted insulin by mouse insulin ELISA kit. For this *in vitro* assay, tolbutamide (200 µM) was used as a standard insulin secretagogue (positive control).

Experimental detail of Fluorescence studies

Ultrospec 4300-pro (GE Healthcare, UK) was used to record UV–visible spectra using quartz cells (10 mm) at approximately 25°C. Spectromax-5 (Molecular Devices, USA) was used to record fluorescence spectra both in dimethyl sulfoxide (DMSO) and water at different concentrations by using 96-well plates (Nunc™, Germany). The data were collected at variable emission and excitation wavelengths (270-600 nm) and the software (SoftMax Pro-V5) was used for analysis.

STATISTICAL ANALYSIS

All statistical analysis was carried out by SPSS 12.0 statistical package (SPSS Inc., Chicago, IL, USA). All values were expressed as mean ± SEM. Statistical difference among groups was assessed by one-way ANOVA with Dunnett's *post hoc* test. To compare data within the group, paired t-tests were conducted. Values were considered to be statistically significant at $p < 0.05$.

RESULTS

Synthesis of crown motifs of type 6 were obtained by following literature protocol (Iqbal *et al*., 2021), which involved the reaction between natural motifs of type (4) and tetraethylene glycol ditosylate (5) in a sealed vessel and inert atmosphere under oil bath heating at 90 °C, for two hours in the presence of powdered K_2CO_3. The reaction afforded globally fair yield for crown adducts 6a-6c. Luckily, during the reaction of chrysin, we got two novel crown ethers chrysin 16-crown-5 (6c) and bischrysin 32-crown-10 (6d) (Scheme 1). The characterization of all synthesized compounds was confirmed by EI and ^{1}H NMR and found in good agreement with the literature (Iqbal *et al*., 2021).

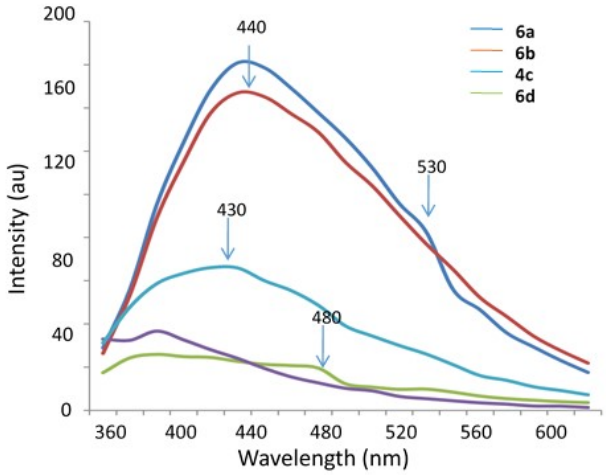


Fig. 4: Electronic emission spectra of natural products and their crown ether derivatives

Antidiabetic Activity

To investigate the antidiabetic potential of synthesized biologically active natural products embedded crown ethers, we performed insulin secretory assay on isolated mice islets according to the standard protocol (Sharma *et al*., 2015). The effects of all pure synthesised crown adducts 6a-6d on secretion of insulin were tested in isolated mice islets.

Table 1: Insulin secretory activity of natural products and their crown ether derivatives[a]

S. No.	Compound	Abs (450 nm)	Insulin* (ng/2islets/h)	Insulin (ng/2islets/h)	Insulin (ng/islet/h)
1	THIQ (4a)	0.111	2.46	9.84	4.92 ± 0.99
2	Biochanin-A (4b)	0.112	2.49	9.96	4.98 ± 0.31
3	Chrysin (4c)	0.106	2.25	9.00	4.50 ± 0.55
4	THIQ 15-Crown-5 (6a)	0.352	8.91	35.64	17.82 ± 1.21
5	Biochanin-A 16-crown-5 (6b)	0.167	4.29	17.16	8.58 ± 1.92
6	Chrysin 16-crown-5 (6c)	0.603	15.55	62.20	31.10 ± 1.56
7	Bischrysin 32-crown-10 (6d)	0.264	6.68	26.72	13.36 ± 0.65
8	Negative control (vehicle)	0.174	5.51	22.04	11.02 ± 0.59
9	Tolbutamide (TB)	0.331	8.01	33.64	16.82 ± 1.91

[a]Mice islets (n = 3) incubated (1 hour, 37 ºC) in KRB buffer containing 17 mM glucose (vehicle) or in the presence of test compounds (200 µM). Values are mean ± SEM from 3 independent experiments from duplicate samples. Statistical difference among groups was assessed by one-way ANOVA with Dunnett's *post hoc* test. Insulin secretory activity by 17 mM glucose alone (vehicle) was expressed as 100%. *represents values at four times dilution.

Table 2: Fluorescent data of natural products and their crown derivatives[a]

S. No.	Compound	Solvent	λ_{max} UV*	λ_{max} exc	λ_{max} em*
1	THIQ (4a)	DMSO	285	290	450
		H_2O	280	285	470
2	Biochanin-A (4b)	DMSO	279 / 365	285 / 370	450 / 440
		H_2O	259 / 350	260 / 355	440
3	Chrysin (4c)	DMSO	281 / 406	285 / 410	430 / 450
		H_2O	272	275	420, 480
4	THIQ 15-Crown-5 (6a)	DMSO	264 / 331	270 / 335	440, 530 / 430
		H_2O	240 / 334	250 / 340	460
5	Biochanin-A 16-crown-5 (6b)	DMSO	264 / 285	270 / 290	430, 480 / 440
		H_2O	256 / 322	260 / 350	440
6	Chrysin 16-crown-5 (6c)	DMSO	268 / 309	270 / 315	420, 530 / 440
		H_2O	264 / 312	270 / 315	420, 480 / 420
7	Bischrysin 32-crown-10 (6d)	DMSO	265 / 309	270 / 315	480, 530 / 420
		H_2O	266 / 329	270 / 335	420, 480 / 430

[a]For UV = 1×10^{-3} M solution and 1 cm path length was used. 1×10^{-5} M DMSO and 1×10^{-3} M water solutions were used to determine emission maximum. *Double maximum for UV and emission were observed except for compound 4a.

The results were calculated as secretion of insulin in ng at the rate of variable number of islets in one hour i.e. ng/2islets/h and ng/islet/h as shown in table 1. The effects of crown adducts on insulin secretion were further compared with their natural product precursors. Interestingly, all the crown compounds showed increased potential for insulin secretion on *in vitro* assay (table 1).

Fluorescence Studies

We also studied the fluorescent potential of the prepared crowns along with their natural product precursors. Wavelength for maximum absorbance (λ_{max}) of all crowns and their precursors were determined in DMSO and water at the concentration of 1×10^{-5} M and 1×10^{-3} M, respectively. Further, relative fluorescence was studied in both solvents as shown in table 2.

DISCUSSION

To investigate the antidiabetic potential of synthesized biologically active natural products embedded crown ethers, we performed insulin secretory assay in isolated mice islets. The effect of all NMR pure crown adducts were tested for their insulin secretory activity and compared with their natural product precursors. All the crown compounds showed increased potential for *in vitro* insulin secretory assay (table 1). THIQ (4a), biochanin-A (4b) and chrysin (4c) showed some extent of inhibition on insulin secretion (4.5-4.98 ± 0.99 ng/islet/h, entry 1-3; table 1) as compared to glucose alone (11.02 ± 0.59 ng/islet/h, entry 8, table 1) which is used as a negative control in this study. Interestingly, bischrysin 32-crown-10 (6d), THIQ 15-Crown-5 (6a) and chrysin 16-crown-5 (6c) showed mild, moderate and strong insulin secretory activity, respectively. Bischrysin 32-crown-10 (6d) could stimulate insulin secretion noticeably (13.36 ± 0.65 ng/islet/h, entry 7, table 1; $P < 0.05$, fig. 3) when compared with the insulin secretory activity by glucose alone. The insulin secretory activity of THIQ 15-Crown-5 (6a) (17.82 ± 1.21 ng/islet/h, entry 4, table 1; $P < 0.01$,

fig. 3) was found significant, very similar to that of tolbutamide (16.82 ± 1.91 ng/islet/h, entry 9, table 1; $P < 0.01$, fig. 3), a standard insulin secretagogue. Chrysin 16-crown-5 (6c) showed potent insulin secretory activity (31.10 ± 1.56 ng/islet/h, entry 6, table 1; $P < 0.001$, fig. 3) that is almost two (02) fold higher than that of standard drug TB (16.82 ± 1.91 ng/islet/h, entry 9, table 1; $P < 0.01$, fig. 3). Biochanin-A 16-crown-5 (6b) showed no activity on insulin secretion. The data suggest that all these crown adducts have different improved mode of action/mechanism on insulin secretion as compared to their un-crowned natural precursors. Further study is under process to explore their insulin secretory mechanism.

Fluorescence labelled molecules (probes) are significant for fluorescence imaging that has become an indispensable tool for envisioning live protein interactions, intracellular networks, diagnosis and revealing biological mechanisms in real time (Li *et al*., 2017). To this consideration, we became interested to study the fluorescent behaviour of the prepared crowns and their natural precursors. All the crown adducts showed hypsocromic shift in absorption spectra upon irradiation with UV light, as compared to their natural product precursors (table 2). In addition, absorbance magnitude also increased for crown analogues as shown in fig. 4. Interestingly, all compounds except 4a showed double maximum for both absorption and emission in water and DMSO. In case of THIQ (4a), emission wavelength 450-470 nm was shifted to 430-530 when crown fragment is introduced (entry 1 *vs* 4, table 2). Crown adducts specially 6a and 6b found highly fluorescent by emitting radiation in visible region of electromagnetic radiations (fig. 4).

CONCLUSION

In summary, various bioactive natural products along with their crown ether analogues investigated for their insulin secretory potential in isolated mice islets as well as for their fluorescent potential. Study showed that insertion of crown scaffold on natural structures resulted significant increase in insulin secretion from mice islets as compared to their natural product precursors. The chrysin 16-crown-5 (6c) showed potent insulin secretory activity (31.10 ng/islet/h) that is almost two (02) fold higher than that of standard drug TB (16.82 ng/islet/h). The data suggest that all these compounds including crowns and their natural product precursors have different mode of action on insulin secretion in mice islets and detailed study would be needed to explore their insulin secretory mechanism**.** Fluorescent examination of crown compounds in general as compared to their natural precursors showed significant increase in absorption and emission maximum (hypsochromic effect). Present optimistic results obtained from this study may be a guided template for developing new effective insulin secretory agents.

ACKNOWLEDGEMENT

This work was supported by the Higher Education commission of Pakistan (Proposal No.6067/Sindh /NRPU/R&D/HEC/2016 & Proposal No. 20–1160/R&D/09).

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