New Synthesized tri-peptide as Inhibitor of krait (Bungarus sindanus) venom acetylcholinesterase

Mushtaq Ahmed (mushtaq213@yahoo.com)
University of Science and Technology

Nadia Mushtaq
University of Science and Technology

Naila Sher
University of Science and Technology

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Abstract

In the current study, the cyclopeptide alkaloid discarine D derived tri-peptides fragments were manufactured and then investigated for their inhibitory potential against krait (*Bungarus sindanus*) venom acetylcholinesterase (AChE) enzyme. The tri-peptides L-Leu-threo-D-Pheser-L-Phe and L-Leu-threo-L-Pheser-L-Phe were chemically synthesized by a conventional method using the benzyloxy carbonyl group for the alpha-amino (α-amino) safety and the methyl esters an amino acids derivative used safety for the carboxyl group. The present paper described that the general synthetic strategy of tri-peptide allows the tri-peptide sequence to be acquired with the N-terminal extreme protected. Kinetic studies using the Lineweaver Burk plot indicated that tri-peptides fragments cause an un-competitive type of inhibition i.e. both $K_m$ and $V_{max}$ values decreased with the increase of tri-peptides fragment concentration (13.5-22.5 µM). The estimated $K_i$ and $IC_{50}$ for krait venom AChE were found to be 17.5 µM and 19.5 µM, respectively. It is concluded from the present paper clarified that the freshly produced tri-peptides fragment can be deliberated as a beneficial mediator for the inhibition of krait venom AChE.

1. Background

The acetylcholinesterase (AChE; E.C.3.1.1.7) enzyme or true cholinesterase is a serine hydrolase existing in both synaptic and non-synaptic tissue (Silman and Sussman, 2005). The main sources of AChE are the brain, muscles, erythrocyte, and cholinergic neurons where its function is revealed in monitoring the various biological measures by a neurotransmitter acetylcholine (ACh) hydrolysis (Chatonnet and Lockridge, 1989; Massoulie et al., 1993; Milatovic and Dettbarn, 1996; Silman and Sussman, 2005).

In nonsynaptic tissue, it is present in snakes venom where its function is unknown (Frobert et al., 1997). In snake, venom is mainly found in the poisonous snake family of the family Elapidae (Frobert et al., 1997). The Elapidae snakes are well known for the extremely toxic constituents in their venom. The Elapidae snake's venom of have numerous toxins, some are enzymatic (enzymes) in nature and others are non-enzymatic (toxin, nerve growth factors, and inhibitors) which are extremely noxious to man, and causing death throughout the world, mainly in Asian countries (Bawaskar and Bawaskar, 2004). The krait (*Bungarus*) genera belong to the family Elapidae which has acetylcholinesterase (AChE) in its venom (Frobert et al., 1997). The AChE enzyme is present in high quantity in the venom of *Bungarus* snake, approximately 8mg/g of dried snake venom (0.8% w/w) having 747000 Ellman's units/g of dry snake venom of AChE-like action (Frobert et al., 1997).

In the venom of krait (*Bungarus sindanus*) the AChE is existing in a monomeric, non-amphiphilic, soluble, form and has a 67 KDa molecular weight (Cousin et al., 1996b). Amino acid sequence analysis indicates that krait venom AChE has very close homology with *Torpedo marmorata*, using the same “catalytic triad” of His, Ser, and for ACh hydrolysis the Glu in the active site (Cousin et al., 1996b).
Up to that time, we found that the krait venom enzyme AChE is highly sensitive to tacrine (Ahmed et al., 2006a). Tacrine is generally used as a therapeutic agent for Alzheimer’s disease treatment (Eagger et al., 1992). Furthermore, the herbicides and pesticides are commonly used as inhibitors of krait venom AChE and exhibited similar behavior as that of human serum butyrylcholinesterase (BChE) (Ahmed et al., 2007). The present study was conducted to synthesize short-chain peptides which can be evaluated as a therapeutic agent for inhibition of krait venom AChE.

2. Methodology

2.1. Materials

Acetylthiocholine iodide (ATC), DTNB [5,5’-dithiobis(2-nitro-benzoic acid)], ethopropazine, and bovine serum albumin (BSA) were acquired from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate (monobasic) and sodium dihydrogen phosphate (dibasic) were acquired from Neon Comercial LTDA (Brasil). All other chemicals used were of methodical grade.

Melting points (mp) were determined by using the MQAPF-301 apparatus. 1H and 13C NMR spectra (nuclear magnetic spectra) were documented on a Bruker Dpx-400 spectrometer operative at the 400.13 MHZ for 1H and 100.62 MHZ for 13C, using tetramethylsilane (TMS) as the interior standard and CDCl₃ as solvent. On pre-coated TLC plates the thin-layer chromatography (TLC) was accomplished (Merck, silica gel 60 F-254). One or more of the following methods were used for the detection of spots: UV (254 nm), o-toluidine, ninhydrin (0.1% in ethanol), and dragendorff’s reagent. the compound’s purification chromatographic was carried out by means of a column packed with silica gel 60 (230–400 mesh) acquired from Merck Co. in system solvents selected by Rf separation in TLC.

All amino acids and substances used, except threo-β-Phenylserine acquired conferring to Shaw and Fox (Kenneth et al., 1953), possess L-configuration, and were commercially available by Aldrich/Fluka, and were utilized without further purification

2.2. Synthesis of dipeptide N-Cbz-L-Leu-threo-D, L-Pheser-OH (3)

To a suspension of threo-D, L-β-Phenylserine methyl ester hydrochloride (2) (10.1 g, 75 mmol), (HOBt) (10.14 g, 75 mmol), N-methyl morpholine (8.24 mL, 75 mmol) and N-Cbz-L-Leu-OH (1) (Figure.1) (19.6 g, 75 mmol) (crude from the previous step) in dry THF (50 ml) at 0°C, followed by addition of DCCI (16.5 g, 80 mmol) stirring at the same temperature for 3 h, the reaction was allowed to warm at room temperature and stirred for additional 20 hrs. Then the mixture of reaction was filtered to remove the DCCU and under the reduced pressure the solvent was removed. The resulting yellow residue was diluted in CHCl₃ (100 mL) and splashed with H₂O (40 mL and 20 mL), with sat. Aqueous NaHCO₃ (2 x 30 mL), solution aqueous 10% citric acid (2 x 30 mL), water (20 mL), with sat, aqueous NaHCO₃ (30 mL) and finally brine (30 mL). The joined layers of organic were dehydrated over MgSO₄. Filtration and concentration afforded
the dipeptide methyl ester that was hydrolyzed in the solution of 25 mL of MeOH and 1M NaOH cooled at 0°C, by flash chromatography the resultant crude oil was cleansed to afford compounds N-Cbz-L-Leu-threo-D, L-Pheser-OH (3) in a total product of 70% as a yellow oil, after purification in flash silica gel column chromatography.

\[ \text{H NMR (CDCl}_3\text{): } \delta 7.22–7.19 \text{ (br s, 10H)}, 6.16 \text{ (br d, 2H)}, 5.73 \text{ (br d, H)}, 4.70 \text{ (br dd, 1H)}, 4.94 \text{ (br s, 2H)}, 4.30 \text{ (br dd, 1H)}, 1.49 \text{ (m, 1H)}, 1.16 \text{ (m, 2H)}, 0.76 \text{ (br d, 3H)}, 0.70 \text{ (br d, 3H)} \].

13C NMR (CDCl3): \( \delta 177.52 \text{ (177.11) (C-9)}, 173.50 \text{ (C-1)}, 156.82 \text{ (156.50) (C-16)}, 140.72 \text{ (C-4)}, 136.03 \text{ (C-18)}, 128.18 \text{ (C-7)}, 127.76 \text{ (C-6)}, 127.67 \text{ (C-20)}, 127.62 \text{ (C-21)}, 125.76 \text{ (C-5)}, 125.76 \text{ (C-19)}, 73.13 \text{ (72.97) (C-3)}, 66.72 \text{ (C-17)}, 59.05 \text{ (C-10)}, 53.41 \text{ (C-2)}, 41.38 \text{ (C-11)}, 24.38 \text{ (C-12)}, 22.54 \text{ (C-14)}, 21.39 \text{ (C-13)}. \]

2.3. Synthesis of tri-peptides Z-L-Leu-threo-L-Pheser-L-Phe-OMe (4) and Z-L-Leu-threo-D-Pheser-L-Phe-OMe (5)

The tripeptide Z-L-Leu-threo-Pheser-L-Phe-OMe production was carried out through our well described procedure (Mostardeiro et al., 2013). 2.36 g (5.53 mmol) of the di-peptide N-Cbz-L-Leu-threo-D, L-Pheser-OH (3) (Figure.1) in 5 mL THF was coupled with L-Phe-OMe (0.91 g, 5.53 mmol) by DCC-method according to Bodansky to give the tri-peptide 78% as a diastereomeric mixture (Bodanszky, 1984). The separation of the diastereomeric mixture was achieved by recrystallization in ethylene ether-petrol ether, yield Z-L-Leu-threo-L-Pheser-L-Phe-OMe (48%) and Z-L-Leu-threo-D-Pheser-L-Phe-OMe (30%), as white solids.

Z-L-Leu-threo-L-Pheser-L-Phe-OMe (4) mp 139.8–141.8 °C: \(^1\text{H NMR (CDCl}_3\text{): } \delta 7.17–7.34 \text{ (br s, 15H)}, 7.09 \text{(d, 1H, J16–10 = 8.0 Hz)}, 7.00 \text{ (d, 1H, J8–2 = 8.3 Hz)}, 5.29 \text{ (d, 1H, J11–10 = 3.0 Hz)}, 5.12 \text{ (br d, 1H)}, 5.10, 5.03 \text{ (d, 2H, J25–25’ = 12.3 Hz)}, 4.77 (dd, 1H, J2–3 = 6.8 Hz, J2–3’ = 4.4 Hz, J2–8 = 8.2 Hz), 4.70 (dd, 1H, J10–16 = 8.0 Hz, J10–11 = 3.0 Hz), 4.11 (m, 1H), 3.72 (s, 3H), 3.10, 3.03 (dd, 2H, J3–2 = 6.8 Hz, J3–2’ = 4.4 Hz), 1.50 (m, 1H), 1.41, 1.28 (m, 2H), 0.85 (d, 3H, J21–20 = 6.4 Hz), 0.85 (d, 3H, J22–20 = 6.4 Hz) (Bodanszky, 1984) C NMR (CDCl3): \( \delta 172.78 \text{ (C-17)}, 171.33 \text{ (C-1)} 169.77 \text{ (C-9)}, 156.25 \text{ (C-24)}, 139.03 \text{ (C-12)}, 135.96 \text{ (C-4)}, 135.79 \text{ (C-26)}, 129.19 \text{ (C-5)}, 128.54 \text{ (C-27)}, 128.52 \text{ (C-14)}, 128.50 \text{ (C-29)}, 128.24 \text{ (C-6)}, 128.00 \text{ (C-28)}, 127.71 \text{ (C-15)}, 127.11 \text{ (C-7)}, 125.86 \text{ (C-13)}, 71.98 \text{ (C-11)}, 67.21 \text{ (C-25)}, 57.76 \text{ (C-10)}, 53.71 \text{ (C-2)}, 53.57 \text{ (C-30)}, 53.32 \text{ (C-18)}, 40.99 \text{ (C-19)}, 37.65 \text{ (C-3)}, 24.54 \text{ (C-20)}, 22.79 \text{ (C-21)}, 21.17 \text{ (C-22)}. \]

FAB-M\(^+\) (m/z): 591

Z-L-Leu-threo-D-Pheser-L-Phe-OMe (5): amorphous powder, \(^1\text{H NMR (CDCl}_3\text{): } \delta 7.48 \text{ (d, 1H, J8–2 = 7.56 Hz)}, 7.10–7.25 \text{ (br m, 15H)}, 6.98 \text{ (d, 1H, J16–10 = 7.9 Hz)}, 5.42 \text{ (br d, 1H)}, 5.31 \text{ (d, 1H, J23–17 = 7.3 Hz)}, 5.04, 4.94 \text{ (d, 2H, J25–25’ = 12.1 Hz)}, 4.50 (dd, 1H, J2–3 = 4.8 Hz, J2–3’ = 7.6 Hz, J2–8 = 7.9 Hz), 4.75 (dd, 1H, J10–16 = 7.9 Hz), 3.99 (m, 1H), 3.61 (s, 3H), 3.08, 3.00 (dd, 2H, J3–2 = 6.0 Hz, J3–2’ = 7.6 Hz, J3–3 = 14.0 Hz), 1.32 (m, 1H), 1.36, 1.30 (m, 2H), 0.72 (d, 3H, J21–20 = 6.4 Hz), 0.72 (d, 3H, J22–20 = 6.4 Hz). 13C NMR (CDCl3): \( \delta 173.50 \text{ (C-17)}, 171.00 \text{ (C-1)}, 171.00 \text{ (C-9)}, 139.40 \text{ (C-12)}, 136.78 \text{ (C-24)}, 135.60 \text{ (C-4)}, 134.95 \text{ (C-26)}, 129.12 \text{ (C-5)}, 128.42 \text{ (C-27)}, 127.92 \text{ (C-14)}, 127.64 \text{ (C-29)}, 127.41 \text{ (C-6)}, 126.99 \text{ (C-15)}, 126.65 \text{ (C-28)}, 126.09 \text{ (C-10)}, 125.79 \text{ (C-13)}, 125.76 \text{ (C-19)}, 73.13 \text{ (72.97) (C-3)}, 66.72 \text{ (C-17)}, 59.05 \text{ (C-10)}, 53.41 \text{ (C-2)}, 41.38 \text{ (C-11)}, 24.38 \text{ (C-12)}, 22.54 \text{ (C-14)}, 21.39 \text{ (C-13)} \).
125.96 (C-13), 125.57 (C-7), 71.80 (C-11), 65.32 (C-25), 58.71 (C-10), 57.17 (C-2), 53.00 (C-18), 52.13 (C-30), 40.76 (C-19), 36.92 (C-3), 24.19 (C-20), 22.20 (C-21), 21.35 (C-22). FAB-M+ (m/z): 591

Scheme 2: tri-peptides Z-L-Leu-threo-L-Pheer-L-Phe-OMe (4) and Z-L-Leu-threo-D-Pheer-L-Phe-OMe (5) synthesis

2.4. Venom

The venom of both female and male snakes was manually squeezed, mixed, lyophilized, and then stored at a low temperature of −20°C for future use. All the female and male krait snakes were captured from the Thatta District of Sindh Province, Pakistan.

2.5. Protein determination

By following the Bradford standard procedure protein was assayed using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.6. Acetylcholinesterase analysis

The activity of AChE was evaluated by the standard procedure of Ellman (Ellman et al., 1961). The rate of hydrolysis rates (V) was determined at different concentrations (0.05–1 mM) of acetylthiocholine iodide (ATC) substrate (S) in 1 mL assay solutions with phosphate buffer (62 mM) at 7.5 pH, and DTNB (0.2 mM) at room temperature 25°C. About 40 mL of diluted snake venom [6 mg of protein] were mixed with the reaction mixture and followed by incubation at 37°C for 10 min. About 0.06 mM Ethopropazine (inhibitor of BChE) was used to inhibit BChE. For determining the inhibitory potential of various concentrations of the tri-peptides fragment (13.5–22.5 µM), the same method and condition were assumed. The reaction of enzyme-substrate (E-S) was started immediately after adding various substrate concentrations. The yellow color development after adding substrate is an AChE activity measurement. The absorbance was examined at 412 nm after an interval of 15 sec for 2 min utilizing the Hitachi 2001 spectrophotometer. All sample solutions operated in replica and repeat at least three times.

2.7. Determinations of kinetic

The IC50 was estimated by the simple plot of % inhibition and % activity vs inhibitor concentrations; while the Ki (inhibitory constant) values were acquired utilizing Cornish-Bowden plots of S/V vs. [I] (Cornish-Bowden and Cárdenas, 1991). The value of K_m (Michaels Manton constant) was acquired employing two different estimates, 1/V vs. 1/S (Lineweaver and Burk, 1934) and V vs. V/S. (Hofstee, 1952; Dowd and Riggs, 1965). The kinetics of the interaction of the tri-peptides fragment with snake venom enzyme (AChE) was obtained by using the Lineweaver Burk (Lineweaver and Burk, 1934), double reciprocal plot to find out the type of inhibition concerning different concentrations of substrate range from 0.05-1 mM. To the reaction mixture these different concentrations of ACh were added at room temperature 25°C after the enzyme (AChE) the mixture was incubated at 37°C for 10 min in the presence and absence of tri-peptides fragments ranging from 13.5–22.5 µM.

2.8. Statistical Analysis
Statistical analysis was accomplished through the Statistica software package (Stat Soft®, TULSA, OK, USA) by means of one-way ANOVA, which was further followed by post-hoc analysis (Duncan multiple range tests).

3. Results

In the present study, the Z-L-Leu-threo-L-Pheser-L-Phe-OMe (4) and its diastereomer Z-L-Leu-threo-D-Pheser-L-Phe-OMe (5) was synthesized as shown in Figure 1. The diastereomeric tripeptide Z-L-Leu-threo-L-Pheser-L-Phe-OMe was found inactive while the tri-peptide fragment Z-L-Leu-threo-D-Pheser-L-Phe-OMe (Figure 1) was found to inhibit the hydrolytic property of krait venom acetylcholinesterase. The IC50, which inhibits 50% of venom AChE was assessed at fixed ATC concentration (0.5 µM) and was found to be 19.5 µM by plotting the % inhibition and % residual activity vs inhibitor concentration (Figure 2).

Furthermore, the result shows that tri-peptides with a threo-D-Pheser unit (Z-L-Leu-threo-D Pheser-L-Phe-OMe), inhibit snake venom acetylcholinesterase in a concentration-dependent mode (Figure 3). Kinetic analyses indicated that tri-peptides fragment caused an un-competitive type of inhibition against AChE (Figure 4) i-e both V_max and K_m decrease with the increase of tri-peptides fragment concentration (Table 1) which is a clear indication of the un-competitive type of inhibition. Moreover, we found that tri-peptides fragment (13.5–22.5 µM) decreased the K_m values from 17–64% whereas 32 to 69.8% inhibition were observed in the V_max values compared with control (Table 1). The estimated K_i value for snake venom AChE was determined by plotting Cornish-Bowden plots of S/V vs. [I] (Table 2).

4. Discussion

Alzheimer's disease (AD) a neurodegenerative is a most common type of dementia is characterized by memory lapses and mental weakening. It is the most common in the old population due to cholinergic neurons loss in selected brain areas due to irreversible deficiency in the formation of the neurotransmitter acetylcholine (ACh). Nowadays therapeutic strategies via Inhibition of AChE are commonly used for AD treatment to increase the amount of ACh which is necessary for normal body function. The amino acid sequence study of venom AChE from different sources show very close homology. They used the same “catalytic triad” of Ser, Glu, and His in the active site of enzyme for substrate (ATC) hydrolysis (Chhajlani et al., 1989; Cousin et al., 1996a). Thus the finding of an inhibitor can be used for multipurpose (Ahmed et al., 2006b) based on quaternary structure, acetylcholinesterase is classified into (a) asymmetric and (b) globular form which contains several sub-units depend on species to species except for snake venom.

The asymmetric form contains two (A8), three (A12), or one (A4) tetrmeric associations of enzyme subunits disulfide that bonded to a single strand of triple-helical collagenic structure subunit. The globular forms (G1, G2, and G4), comprises one, two, or four catalytic subunits, and exhibits similar catalytic property (Silman and Futerman, 1987; Massoulié et al., 1999).

In snake venom, acetylcholinesterase is present in a monomeric form which is highly active compared to other sources. In snakes, krait (Bungarus) venom contains a very large amount of AChE which has a very potent hydrolytic property (Frobert et al., 1997).
In the present study, the Z-L-Leu-threo-L-Pheser-L-Phe-OMe (4) and its diastereomer Z-L-Leu-threo-D-Pheser-L-Phe-OMe (5) was synthesized as shown in Figure.1. The diastereomeric tripeptide Z-L-Leu-threo-L-Pheser-L-Phe-OMe was found inactive while the tri-peptide fragment Z-L-Leu-threo-D-Pheser-L-Phe-OMe (Figure.1) was found to inhibit the hydrolytic property of krait venom acetylcholinesterase in vitro using test tube assay based on Ellman's 15 methods with some modification (Ahmed et al., 2006a; Ahmed et al., 2007). The IC50, which inhibits 50% of venom AChE was assessed at a fixed ATC concentration (0.5 µM) and was established to be 19.5 µM by plotting the % inhibition and % residual activity vs inhibitor concentration (Figure. 2). Furthermore, the result shows that tri-peptides with a threo-D-Pheser unit (Z-L-Leu-threo-D Pheser-L-Phe-OMe), inhibit snake venom acetylcholinesterase in a concentration-dependent mode (Figure. 3), while its stereoisomer with a threo-L-Pheser, was inactive. Kinetic analyses showed that the new tri-peptides fragment exhibited an un-competitive type against AChE inhibition (Figure. 4) i.e both $V_{max}$ and $K_m$ decrease with the increase of tri-peptides fragment concentration (Table.1) which is a clear indication of the un-competitive type of inhibition. Moreover, we found that the tri-peptides fragment (13.5–22.5 µM) decreased the $K_m$ values from 17–64% whereas 32 to 69.8% inhibition was observed in the $V_{max}$ values compared with control (Table.1). The estimated $K_i$ value was calculated for snake venom AChE by means of Cornish-Bowden plots of $S/V$ vs. $[I]$ (Table.2).

5. Conclusion

It is concluded from the present study that the easy synthesized new tri-peptides fragment can be considered as an inhibitor of krait venom AChE. Furthermore, this finding opens a new area of research to investigate the inhibitory action of this simple tri-peptide fragment against another source of acetylcholinesterase to consider this synthetic peptide as a therapeutic agent for the AD treatment. The present outcomes suggested that the stereochemistry of the β-phenylserine residue in the tripeptide structure influences bioactivity.

Declarations

Conflict of Interest

We are pleased to submit our paper. We do not have any conflict of interest.

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Scheme

Scheme 1 and 2 are available in the Supplementary Files section.

Tables

Table 1

Effect of tri-peptides fragments on $K_m$ and $V_{max}$ of Bungarus Sindanus (Krait) venom AChE.

| Tripeptides fragment (µM) | $K_m$ (mM) | % Decrease | $V_{max}$ (µmol AcSCh/min/mg protein) | % Decrease |
|---------------------------|------------|------------|--------------------------------------|------------|
| 0                         | 0.317      | 0          | 434.95                               | 0          |
| 13.5                      | 0.263      | 17.03      | 294.85                               | 32.2       |
| 18                        | 0.161      | 49.2       | 182.66                               | 58.0       |
| 22.5                      | 0.114      | 64.03      | 131.33                               | 69.8       |

Table 2

$IC_{50}$ was calculated from the simple plot of % activity and % inhibition vs. $[I]$. The values were obtained for at least three experiments while the $K_i$ calculated for snake venom AChE was obtained by using Cornish-Bowden plots of S/V vs. $[I]$. 

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|               | $K_i$ (µM) | $IC_{50}$ (µM) |
|---------------|------------|---------------|
| Tri-peptides fragment | 17.5       | 19.5          |

**Figures**

**Figure 1**

Synthesis of the tripeptides N-Cbz-L-Leu-threo-D and L-Pheser-L-Phe-OMe. Reagents and conditions: (a) DCC, HOBt, NMM, THF, 0°C; (b) NaOH 1M, MeOH , 25°C; c) L-Phe-OMe, DCC, HOBt, NMM, THF, 0°C.
Figure 2

Snake venom acetylcholinesterase. A plot of % residual activity and % residual inhibition vs tri-peptides fragment concentration.
Figure 3

Inhibition of Krait (*Bungarus sindanus*) AChE in the presence of various doses of the synthesized tripeptides fragment. The rates of hydrolysis V were dignified at 412 nm by using a fixed ACh concentration of 0.5 mM (AcSCh) in 1 mL assay solutions with phosphate buffer (62 mM and pH 7.4) and 0.2 mM of DTNB followed by the incubation at 37°C for 10 min before adding of the substrate.

\( (n = 4), \ p < 0.05. \)
Figure 4

Krait Venom AChE experiments in the absence and presence of various doses of the tri-peptides fragment as shown in the legend box. All experiments were repetitive at least four times and in all cases, comparable results were acquired.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- Scheme2.png