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In silico designing of DASM with Pyrazofurin, a possible drug candidate for Anthrax

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Abstract

Bacillus anthracis is a notorious occupational zoonotic agent causing anthrax to veterinarians. Anthrax infection is highly lethal in its most virulent form and produces a combination of three endotoxins namely Protective antigen, Lethal factor and Edema factor. Protective antigen binds to target cells and eases the transfer of either Edema factor or Lethal factor into the cytosol. One possible protein target is Furin, an endogenous, membrane-associated, trypsin-like serine endoprotease which is utilized by *B. anthracis* as a means of activating Protective Antigen (PA). In this study, for the purpose of lead development, Dehydro Andrographolide Succinic acid Monoester (DASM) inhibitor of Furin was selected as a template. Furin is involved in other diseases, DASM was modified by adding anti-cancer, anti -inflammatory, anti-tuberculosis and anti-viral groups. It was found that modification with each group like Pyrazofurin, Ethanol, Dimethyl, Dimethylbutyl, Mercaptopurine, Sulfacetamide, Ethambutol, Isoniazid to DASM showed a better interaction. The ligand with Pyrazofurin as the modification group showed high affinity with Furin involving the active site residue SER 368. The results suggest that DASM containing pyrazofurin (compound2) as side group can be an appropriate lead for the development of Furin inhibitors.

Keywords: *Bacillus anthracis,* Dehydro Andrographolide Succinic acid Monoester, Furin, Protective antigen, Lethal factor

1. Introduction

Anthrax is a serious disease caused by the vector bacteria *Bacillus anthracis* [1-3]. The disease can spread as a communicable one and cause increased mortality [1-3]. Another dimension is that anthrax spores can be infused as required by people with vested interests and can be used as a bio-weapon [4]. Developing new drugs to treat anthrax and other biological infectious agents has now become a major research concern [5]. Human beings are affected by anthrax when they come in contact with infected animals or their products (such as skin and meat). Anthrax spores can enter the host animal through inhalation and reach the regional lymphatic tissues in the mediastinum [6]. These spores then germinate and secrete Anthrax toxin (Atx) [7] which is made up of three proteins: Protective antigen (PA), Lethal factor (LF) and Edema factor (EF) [8,9]. Protective antigen (PA) binds to anthrax toxin receptor and assists in the entry of toxic enzymes LF and EF into the target cells [10]. This requires the precursor form of PA (83kDa) to be cleaved to a functionally active PA (63kDa) by furin [11] or furin family proteases [12]. The functionally active form of PA then heptamerizes and forms seven member pore structures on the cell membranes [13-15] and act as delivery channels through which either EF or LF can enter the cytosol of target cells [16-19]. LF is a zinc-dependent metallo-enzyme which causes the proteolysis of members of host cell mitogen-activated protein kinase kinases (MAPKKs) [20,21]. MAPKK is believed to be fundamental for the maintenance of viability of macrophages, monocytes and dendritic cells [13]. The mitogen activated protein kinase/extracellular signal regulated protein kinase (MAPK/ERK) pathway helps in communicating the extracellular signals to the nucleus and adaptation of the cell to the environment [20, 22]. The delivery of LF into the host cell causes a rapid loss of viability [13]. MAPKKs reduce in numbers preventing p38 kinase mediated activation of immune mechanisms and help *B. anthracis* to evade host immunity[20, 21,

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23]. While PA, LF and EF are individually not toxic, the combination of PA and LF becomes Lethal toxin (LT) which can alter the physiology of the cells and causes death [24]. PA combines with EF to form Edema toxin (ET) leading to tissue swelling and may also result in death [24, 25].

Among the many potential targets available for the therapeutic intervention against anthrax lethal toxin [17, 26], using inhibitors of furin appears to be a promising strategy [11, 14]. Furin is a membrane-anchored, calcium dependent serine protease and a member of the proproteinconvertase (PCs) family [12, 27]. It converts precursor proteins into their functionally active forms[12, 27]. Since proteolytic cleavage of anthrax PA by furin [11] is an obligatory step for the entry of the active components of toxin LF [28] and EF [29] into the cytosol of host cells, inhibition of Furin offers an attractive therapeutic approach to combat anthrax. It has been shown that anthrax toxicity can be attenuated by Furin inhibitors [30]. Apart from anthrax, Furin is also involved in various other diseases like cancer, virulence of many viral pathogens, activation of bacterial toxins [31] and various inflammatory diseases [32].

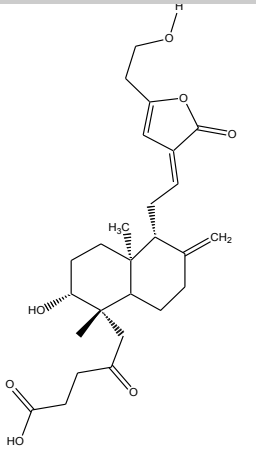
One of the approaches followed in the present study was that of finding a suitable molecule designed to inhibit the Furin through *in silico* drug designing technique. To achieve this, a base molecule which has proved efficacy like DASM (Dehydro Andrographolide Succinic acid Monoester) was considered. DASM is a natural plant extract of *Andrographis paniculata* having protease inhibitory property [32] and is said to have anti-HIV properties [33] as well as Furin inhibition [34]. DASM was modified by adding various chemical groups to generate new inhibitors. These groups included anti-cancer (eg: Bis (chloro ethyl) nitrous urea (CENU), Pyrazofurin, Ethanol, Dimethylbutyl, Thiopurine, Dimethyl, Guanazole, Butyl), anti-tuberculosis (eg: Ethambutol, Isoniazid), anti-inflammatory (eg: Sulfacetamide) and anti-viral (eg: Stavudine, Emtricitabine, Lamivudine) molecules. The rationale behind using these groups was to target broad category of diseases like cancer, viral and inflammatory diseases (which involves furin) apart from targeting anthrax. By logical and analytical rationale, functional groups having potential to achieve ultimate inhibiting action was attempted. The present study revealed that the andrographolide derivatives are effective Furin inhibitors. DASM with pyrazofurin as the modification group showed better interaction with Furin than other derivatives. Hence this compound can be considered for therapeutic intervention against Anthrax.

2. Materials and methods

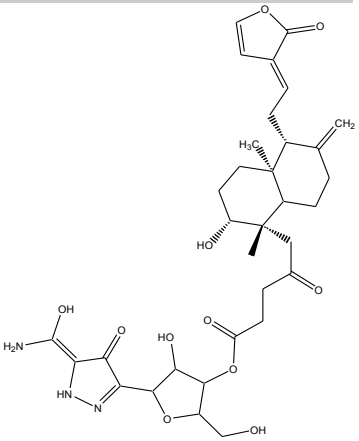
2.1 Preparation of ligand structures

DASM was chosen as the base molecule and modified by adding bio active side group/chain. All the ligand structures were developed and energy minimized using CambridgeSoft ChemOffice 6.0 (CambridgeSoft.com, Cambridge, MA, USA) tool. The small-molecule topology generator Dundee PRODRG 2 server [35] is used for ligand optimization. The structures of the ligands obtained after modification using DASM as the template are given in table-1.

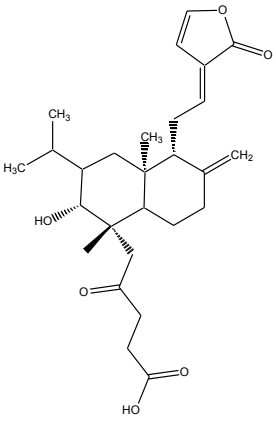
Table-1: Structures of modified ligands



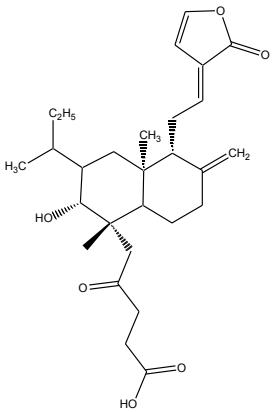
DASM with Ethanol (compound1)



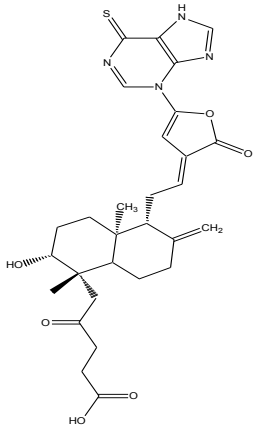
DASM with Pyrazofurin (compound2)



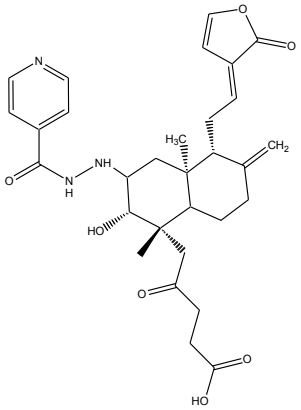
DASM with Dimethyl (compound3)



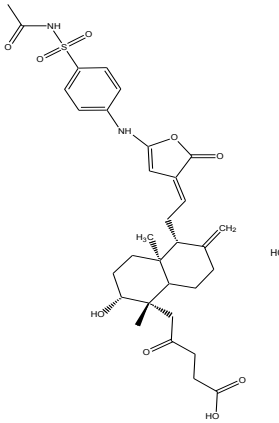
DASM with Dimethylbutyl (compound4)



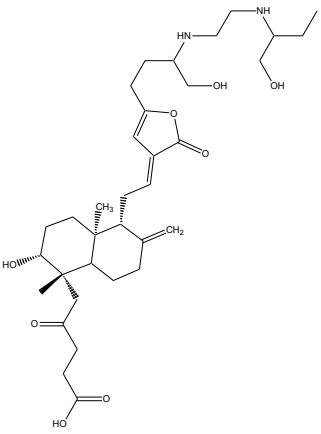
DASM with Mercaptpurine (compound5)



DASM with Isoniazid (compound6)



DASM with Sulfacetamide (compound7)



DASM with Ethambutol (compound8)

2.2 Preparation of protein structure

X-Ray Crystallographic structure of the 2.6Å model of the N-terminal domain of the proproteinconvertasefurin (PDB ID: 1P8J) was obtained from the protein databank (www.pdb.org). The structures were edited by deleting all the HETATOMS, water molecules and co-crystallized compounds. The active site residues include Asp 154, Asp 191, Asn 192, His 194, Leu 227, Val 231, Glu 236, Ser 253, Trp 254, Gly 255, Pro 256, Glu 257, Asp 258, Asp 264, Ala 292, Ser 293, Gly 294, Asn 295, Asp 306, Tyr 308, Thr 309 and Ser 368 of A chain [36].

2.3 Molecular docking

AutoDock 4.0 program [37] was used for docking ligands to the active site of Furin. Topology file and other force field parameters were generated for all ligands using the PRODRG server. Flexible torsions for all ligands were defined using AUTOTORS. The docking site for all ligands on 1P8J was defined at the position of the co-crystallized ligand by using PyRX 0.8 interface[38] with box size of 63x83x70, spacing of 0.375, grid centre 43.99, -5.40 and 120.88 and assigning complete Degrees of Freedom. The Lamarckian Genetic Algorithm (LGA) was employed with the population size of 150 individuals, maximum number of generations and energy evaluations of 27,000 and 2.5 million respectively. From the estimated free energy of ligand binding (ΔG), the inhibition constant (K_i) for each ligand was calculated. Only the best pose (the one with the lowest binding energy) was considered for each ligand and analyzed for protein-ligand interaction using Ligplot$^+$ [39]. The co-ligand Decanoyl-ARG-VAL-LYS-ARG-Chloromethylketone and DASM were taken as reference ligands to compare and assess the performance of the ligands (obtained by modifying DASM) against Furin. AutoDock results were analyzed in MGL tools [40].

2.4 Pre-Molecular Dynamics processing

Protein was prepared by adding hydrogens and AM1-BCC partial charges using UCSF chimera [41]. Protein was defined using Amber-99SB force field parameters [42]. Ligands were defined using Generalized Amber Force Field (GAFF) parameters [43] and AM1-BCC partial charges were added using ANTECHAMBER [44] followed with conversion to GROMACS compatible topology using ACPYPE [45].

2.5 Molecular Dynamics Simulation

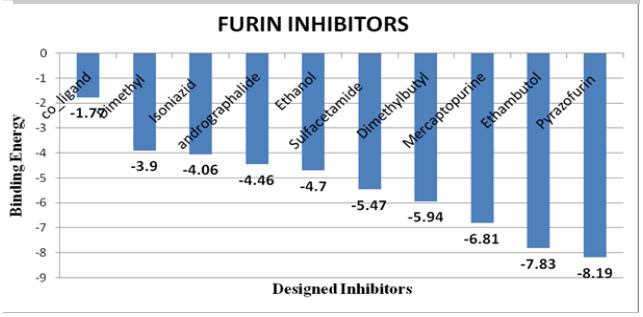
MD simulation was performed using GROMACS version 4.5.5 compiled in single-precision mode [46, 47]. The complex (compound 2 bound to active site of Furin) was subjected to 10ns simulation. A simulation cell was created in a cubic periodic box with a minimum distance of 1nm between the protein and the box walls. The complex was bathed with TIP3P water molecules along with appropriate number of Sodium ions to neutralize the system. Energy minimization for the complex run was performed by using 50000 steps of steepest descent coupled with conjugate gradient method at every 100 steps or until the maximum force was smaller than 100 kJ $mol^{-1}nm^{-1}$. The position restrained run for 300 ps was carried out to allow the randomization of water molecules around the complex, followed by 10 ns isobaric-isothermal ensemble simulation. Particle-Mesh Ewald method (PME) was used to calculate long range electrostatic interactions with cut off for distance as 1nm. The dispersion interactions, both short-range repulsive and attractive, as described by Lennard-Jones, had a cut-off at 1nm. The LINCS algorithm was used to constrain bonds during the position restrained run for 300ps. At every 10 steps, neighbour searching was carried out. A Parrinello-Rahman barostat pressure of 1bar was used with a coupling constant of Tau_P = 0.5ps and compressibility of 4.5e-5 (bar^{-1}). Complex, water and ions were coupled to the thermal bath at 300 K, using a v -rescale coupling constant Tau_T = 0.1 ps.

3. Results and discussion

3.1 Molecular docking

The docking results reveal that the binding energy of co-ligand (Decanoyl-ARG-VAL-LYS-ARG-Chloromethyl ketone (Dka801)) is -1.77 kcal/mol and the reference compound Andrographalide is -4.46 kcal/mol. The modified andrographalide compound with different functional groups showed varied binding energies. Graphical representation of binding energy comparison is given in Table-2. In this group of compounds, Dimethyl and Isoniazid shows higher binding energy when compared to parent molecule, andrographilide, but it is slightly less than that of the co-ligand. The modified ligands having side-groups as Ethanol, Sulfacetamide, Dimethylbutyl Mercaptopurine, Ethambutol and Pyrazofurin have the binding energy -4.7, -5.47, -5.94, -6.81, -7.83 and -8.19 kcal/mol, respectively. The result clearly indicates that Pyrazofurin is better inhibitor in comparison to co-ligand and andrographolide, the parental molecule used as reference. The further docking results show 6 hydrogen bonds with the receptor protein involving Leu227, Gly229, Gly255, Asp258 and Asp306. Active site residues like Asp191, Asn192, Asp228, Val231, Trp254, Pro256, Gly294 and Asn295 showed the hydrophobic interaction with pyrazofurin. The most favourable conformation resulted from the docking of pyrazofurin into the active site of Furin is similar to that of Dka801 as shown in Figure 1a and 1b. Oxygen of fragment 3 interacted with Delta 2 Oxygen of Asp306 with bond length 2.84Å, Two nitrogens (NBU and NBD) of fragment 3 interacted with Delta 1 & Delta 2 Oxygen of Asp258 and show the bond length of 2.78Å & 2.92Å respectively. Oxygen (OBT) of fragment 3 interacted with nitrogen of Gly255 with bond length of 2.89Å. The

Table-2: Comparison in Binding Energy of different ligands against Furin using AutoDock 4



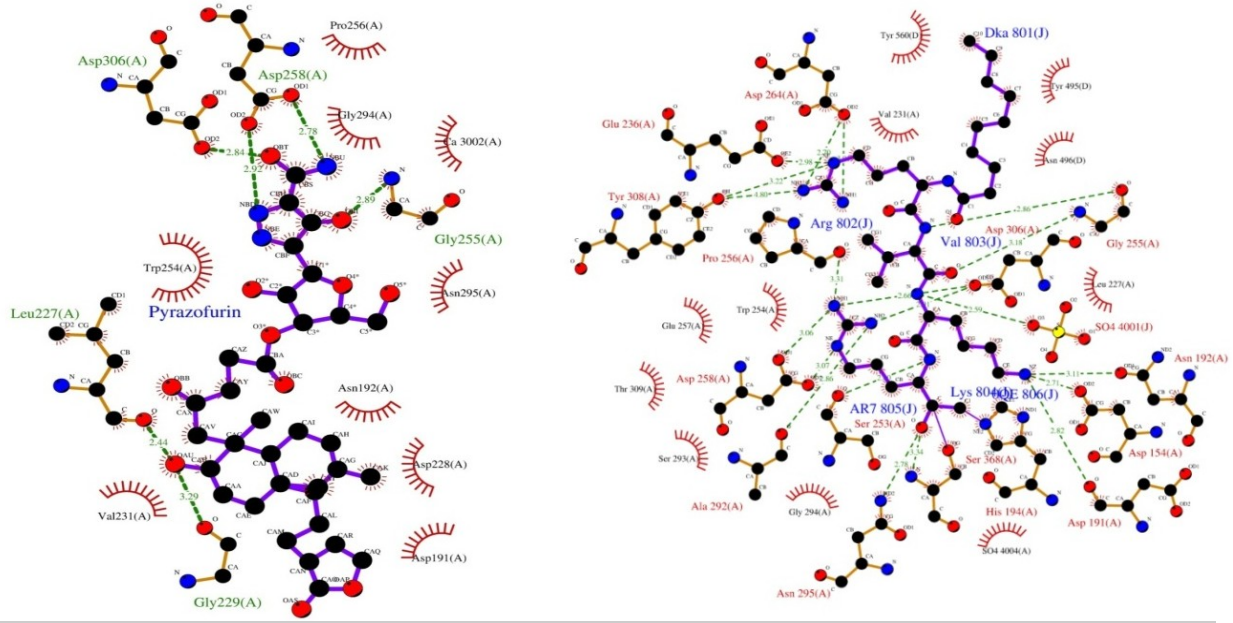


Figure-1: Interaction analysis of Furin with a) Pyrazofurin b) co-crystallised ligand Dka801

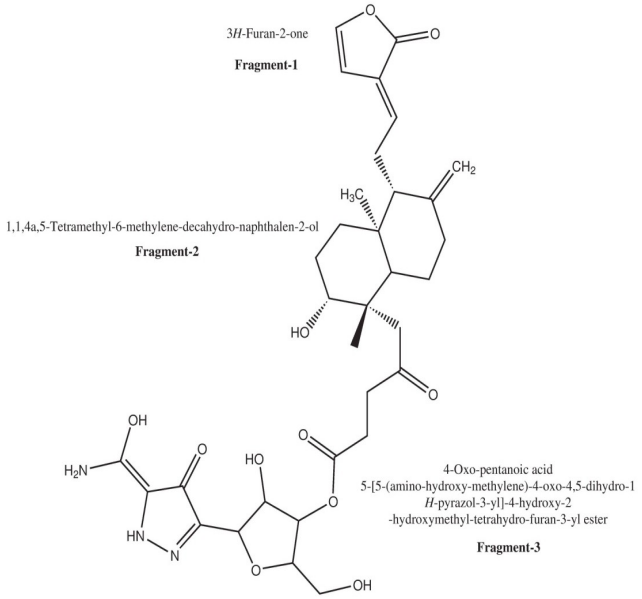


Figure-2: Representation of fragments in compound2.

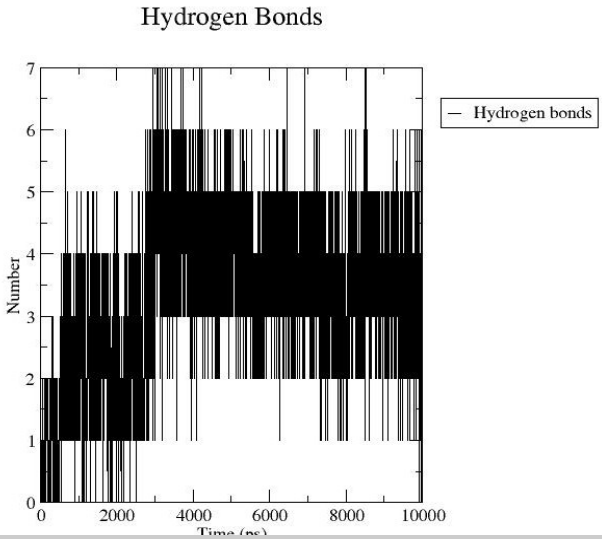


Figure-3: Number of intermolecular hydrogen bonds involving Furin with compound2 during 10ns trajectory

hydroxyl group of fragment 2 interacts with oxygen of Leu227 with bond length 2.44Å and oxygen of Gly229 with bond length 3.29Å.

3.2 Analysis of Molecular Dynamics Simulation

Plot for intermolecular hydrogen bonds involving protein and inhibitor is shown in Figure-3. Analysis of the intermolecular hydrogen bonds during the 10ns simulation shows that for most of the trajectory at least 5 intermolecular hydrogen bonds are observed, which infers the stability of the interactions. The distance between the active site residue Trp254 and ligand was observed to be below 0.8nm during 10ns simulation (Figure-4) and reveals that the ligand was stable in the active site. After simulation for 10ns, we observe that active site residues such as Trp254, Gly255, Thr365 and Ser368 showed hydrogen bond formation and residues His194, Leu227,

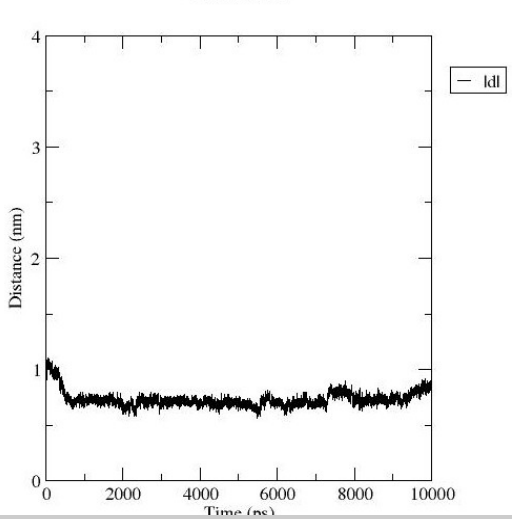


Figure-4: Distance plot of the ligand from the active site residue Trp-254 during 10ns trajectory

Ser253 and Asn295 showed hydrophobic interaction (Figure-5)

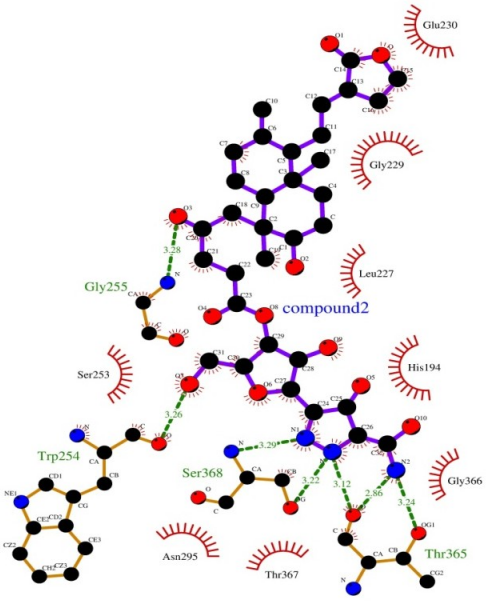


Figure-5: Hydrogen bond and hydrophobic interaction of ligand in the active site after simulation for 10 ns.

4. Conclusion

Furin is a biological target for a wide range of diseases since it is responsible for infectivity or survival of various bacterial and viral pathogens. The DASM, a natural plant extract of *Andrographis paniculata* was used as reference molecule and modified by adding anti-cancer, anti-inflammatory, anti-tuberculosis and anti-viral groups. After modification of the Andrographolide derivative, the docking results have shown good interactions between Furin and the modified ligands making the modified DASM as a potential Furin inhibitor. The binding energies are also shown to be minimal for few ligands compared to naturally bound co-crystal and DASM. Out of all the conformations obtained after the docking runs, compound2 showed the best interaction with Furin in terms of binding energy, hydrogen bond formation and hydrophobic interactions. Molecular dynamics study indicates the stability of compound2 in the active site for 10ns suggesting that it has the potential to act as a lead molecule for the treatment of anthrax. Since DASM has been modified using anti-cancer, anti-tuberculosis, anti-inflammatory and anti-viral groups, the inhibitors may target a wide variety of diseases apart from targeting anthrax alone. Further chemical synthesis and animal trials followed by biopharmaceutical scale up feasibilities could be encouraged. .

Conflict of interest

The author's declares none.

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