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# *Rhodobacter sphaeroides MSB 57* - A natural source of 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl) : A novel inhibitor for reverse transcriptase of HIV

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**ABSTRACT**

*Drug discovery and development is an intense, lengthy and an interdisciplinary endeavour. Drug discovery is mostly portrayed as a linear, consecutive process that starts with target and lead discovery, followed by lead optimization and pre-clinical* in vitro *and* in vivo *studies to determine if such compounds satisfy a number of pre-set criteria for initiating clinical development. Scope of the present work is to find out drug leads from a natural resource, marine sponge associated bacteria. Secondary metabolites were produced using tryptic soy broth and the conditions were optimized. Compounds were extracted by ethyl acetate and purified by preparative TLC. Unknown compounds were further identified by GC/MS analysis. Drug likeliness was studied by Docking and ADME/TOX. Based on the Insilco work, target for the compound 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl) was found to be reverse transcriptase of HIV. Maximum passive absorption was 100% ,contribution from transcellular route(99%) & Paracellular route (1%). Molecular Weight was 180.25 Number of Hydrogen Bond Acceptors were 3 & number of rotatable bond was one. Log P value was 1.99 and AMES Test score was 0.026. Hence the compound obeys Lipinski's thump rule of five and proves to have drug likeliness.*

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## INTRODUCTION

Over the last three decades, the methods for discovering new drugs have tremendously developed. Chemistry, pharmacology, microbiology, and biochemistry helped to shape the course of drug discovery and to bring it to a level where new drugs are no longer generated solely by the imagination of chemists but result from a direct dialogue between biologists and chemists. More recently, the advent of genomic sciences, rapid DNA sequencing, combinatorial chemistry, cell based assays, and automated high throughput screening (HTS) has led to a "new" concept of drug discovery. In this new concept, the critical interplay between chemists and biologists and the quality of scientific reasoning are implemented by the possibility of analyzing large numbers of data. Large numbers of compounds are now readily tested in computer-based *in silico* screening and then assayed in biological assays to confirm/disprove their biological activity, with great saving of time and money. Such "hits" - compounds that elicit a positive response in a particular biological assay - are then further developed to "leads", i.e., compounds that continue to show the initial positive response in more complex models in a dose-dependent manner.

The identification of a good pre-clinical candidate is than followed by its development into a clinical candidate and its clinical development. The "perfect " drug is typically required to satisfy these three points: i) it must be safe; ii) it

must be effective in treating the specific disease for which it was designed; iii) it can be manufactory in a clean and reproducible process. At the end of the four phases of the clinical development the drug is finally ready to be safely used by the patients. In the last two decades, the wide distribution of the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the acquired immune deficiency syndrome (AIDS), a global epidemic which has become a major public health problem worldwide, has dramatically turn the attention of scientists to drug development programs aimed to the inhibition of this retrovirus. In fact, given the great difficulties in developing an HIV effective vaccine, in the last 20 years major efforts have been addressed towards the identification of valuable therapeutic strategies. Their development is a very successful story that has turned a dreadful disease into a manageable chronic infection, at least for the patients who can somehow access the therapy [1].

## MATERIALS AND METHODS

### PURIFICATION OF BIOACTIVE COMPOUNDS

Tryptic digest broth was prepared and optimum conditions were maintained to produce maximum yield and wide activity. Following inoculation with s *Rhodobacter sphaeroides* MSB 57, it was incubated in a shaker at 25 ºC for 72 hours. It was centrifuged at 10,000rpm for 10 minutes, the supernatant was filter-sterilized (0.2µm pore-size filter), heated at 85 ºC for 10minutes, and stored at 4ºC until use.

### SOLVENT EXTRACTION

Cell free extract was acidified using 0.1N HCL and various solvents such as n-Butanol, Methanol, Hexane, Chloroform: Methanol(2:1) and Ethyl acetate were used to extract the bioactive compounds . Solvent and cell free extract was taken in 1:1 ratio in a separating flask and which was shaken vigorously for 30 minutes and kept in a burette stand for 1hour. The organic phase was separated and their ability to inhibit the growth of test isolates were studied.

### LAMBDA MAX DETERMINATION OF CRUDE EXTRACT:

Nanovue was used for the determination of lambda max of the compounds in the crude extract using multiple wavelength option. Various wave lengths from 240nm – 520nm were used and the maximum absorption rate was determined in terms of optical density.

### QUALITATIVE THIN LAYER CHROMATOGRAPHY:

TLC plates were washed and activated at 100ºC for 1 hour. Slica gel slurry was prepared and poured on the glass slide and spreaded evenly using spreader and kept in hot air oven for 45 minutes. The extracted secondary metabolites were spotted on the TLC plate. The prepared solvent contains chloroform and acetic acid the ratio of 9: 1 by volume. The solvent was poured in a buffer tank and it was allowed for saturation for 30 minutes. The TLC slide was placed in the beaker at one end of the slide immersed in the solvent prepared. It was eluted for 30 minutes until the solvent migrates up to three fourth of the plate. One plate was kept into a beaker containing iodine crystals for identification. One was applied with Ninhydrin and the other was examined under UV transilluminator.

### GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY:

The GC/MS analyses were carried out on GC/MS [JEOL GCMATE II GC-MS] system equipped with a quantitative analysis by SIM mode detector (Indian Institute of Technology) (SAIF- Chennai) . A VF-5ms column of 30m length, 0.25mm diameter, and 0.25µm film thickness was used. The oven was programmed from an initial temperature 70 ºC (hold for 2 minutes) to the final temperature 300 ºC at the rate of 10(35.0minutes). The final temperature hold up time was 10 minutes. Helium at the rate of 1 ml/min was used as the carrier gas in constant flow mode. The inlet and interface temperatures were kept at 2800 ºC . The EI source was operated at 2300 ºC and the quadruple temperature was 5000 ºC . The MS was scanned from 1-3,000 m/z. One micro liter of the sample was injected in split mode at a split ratio of 40. WILEY library search and NIST library search were used for compound identifications.

### Preparation of ligand

The ligand compound was drawn using ACD/ Chemsketch (12.0) (1) and saved in mol 2 format. The saved ligand compound was later imported and minimized in Argus Lab after adding hydrogen bonds. The molecule thus obtained was saved in PDB format.

**Argus Lab**
Argus Lab is an electronic structure program that is based on the quantum mechanics; it predicts the potential energies, molecular structures, geometry optimization of structure, vibration frequencies of co-ordinates of atoms, bond length, bond angle and reaction pathway (Cheng, 2003). The energy (E) of the molecule is calculated as E= E stretching + E bending + E torsion + E Vander Waals + E electrostatic + E hydrogen bond +cross term. These terms are of importance for the accurate calculation of geometric properties of molecules. The set of energy functions and the corresponding parameters are called a force field[1].

**Genetic algorithm**
Genetic algorithm (GA) is a computer program that mimics the process of evolution by manipulating a collection of data structures called chromosomes. It is also stochastic optimization methods and provides a powerful means to perform directed random searches in drug designing [9]. It study properties of QSAR, utilizes the novel representation of the docking process, each chromosome encodes an internal conformation and protein active site and includes a mapping from hydrogen-bonding sites in the ligand and protein (Seiburg, 1990; Rajasekhar et al., 2010). On decoding a chromosome, fitness is evaluated by PLS (partial least squares) cross validation to position the ligand within the active site of the protein, in such a way that as many of the hydrogen bonds suggested by the mapping are formed (Kimura et al., 1998). Docking of flexible ligands to macromolecules is paramount in structure based drug design, few programs that work with GA also enable automated docking; another application of GA is the automated generation of small organic molecules using lipophilicity, electronic properties and shape related properties for calculation of the scoring function [6].

**Docking using GOLD**
Genetic algorithm was implemented in GOLD v 3.2 that was applied to calculate the possible conformations of the drug that binds to the protein (Selvaraj, 2008). The genetic algorithm parameters used are population size-100, number of islands-5, niche size-2, selection pressure-1.1, migrate-2, number of operators-100,000, mutate-95, cross over-95. During docking process, a maximum of 10 different conformations was considered for the drug. The conformer with highest binding score was used for further analysis[4].

**ADME/ toxicity testing**
ADME (absorption, distribution, metabolism, and excretion) determines drug like activity of the ligand molecules based on Lipinski rule of 5 (Konstantin, 2005). Increasing clinical failures of new drugs call for a more effective use of ADME/TOX technologies, becoming more advanced and reliable in terms of accuracy and predictiveness, an increase in their usage is expected during the initial development and screening phase of innovative drugs. New computational methods including consensus modeling show promise for increase in the accuracy of Insilco ADME-TOX prediction used for virtual screening in lead optimization.

## RESULTS AND DISCUSSION

**MEDIA OPTIMIZATION**
There were three different media used for the production of secondary metabolites. Their biomass production ability was studied. All the three media were slightly modified in order to grow the sponge associated bacteria**.** *Rhodobacter sphaeroides MSB 57* had also showed higher yield in Tryptic digest broth. It was 78.6% higher than Zobell marine broth and 86.1% higher than Brain heart infusion broth (Figure : 1).

**STRAIN IMPROVEMENT**
UV mutation was accomplished to improve the product yield of the sponge associated bacteria. After mutation both primary and secondary metabolite production ability was studied, which gave a characteristic result. There were no yield improvement in terms of secondary metabolites but their proteolytic activity was drastically increased. There was a 66.2% reduction in secondary metabolite yield in *Rhodobacter sphaeroides MSB 57* after mutation(Figure : 2).

**PROCESS OPTIMIZATION**
Various parameters were optimized in order to increase the yield and efficacy of the secondary metabolites. There were four different incubation periods used. They are 48 hrs, 72 hrs, 96 hrs and 120 hrs. Among them 72 hrs showed maximum activity. pH optima was studied starting from 6.5 to 9.0 with 0.5 difference, among them pH 8.5 showed maximum activity. Temperature optima was determined with ranges from 15 ºC to 40 ºC, among them 25 ºC was

found to be an optimum temperature. Influence of agitation during incubation for the production of secondary metabolite was studied with an agitation speed ranging from 50 rpm to 200 rpm. The maximum product formation was determined at 150 rpm. The substrate soy bean meal concentration was optimized and which was found to be 0.5% with a maximum yield.

**SOLVENT EXTRACTION METHOD FOR THE RECOVERY OF BIOACTIVE COMPOUNDS:**
Solvents such as n-Butanol, Methanol, Hexane, Chloroform : Methanol (2:1) and Ethyl acetate were used to extract bioactive compounds from the selected bacteria. Among the five solvents studied ethyl acetate was found to be a suitable solvent.

**Figure : 1**

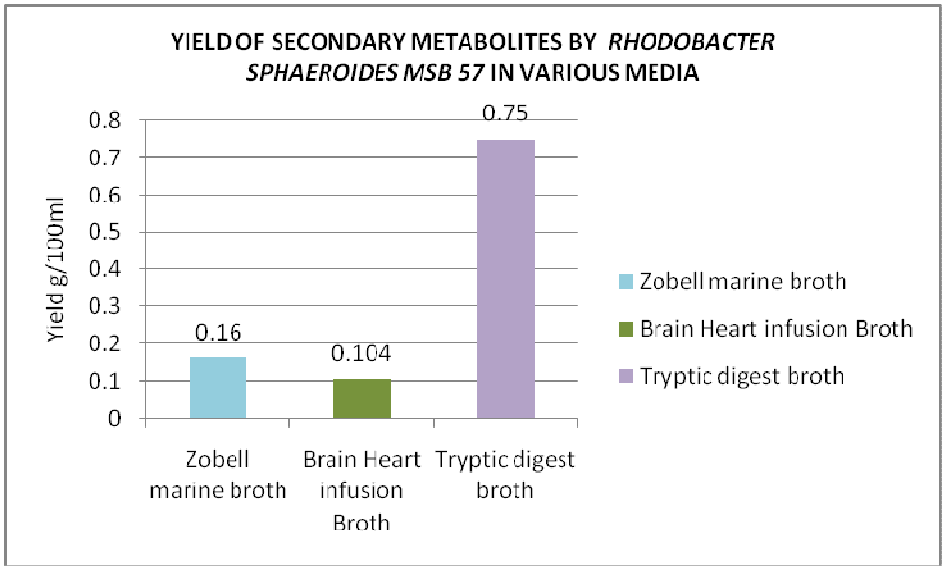



**Figure : 2**

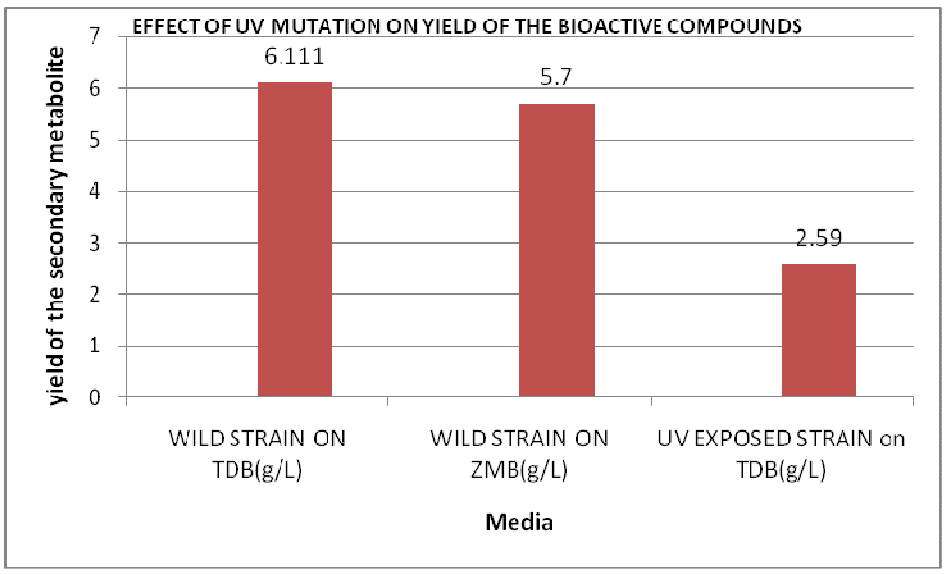


**Figure : 3**

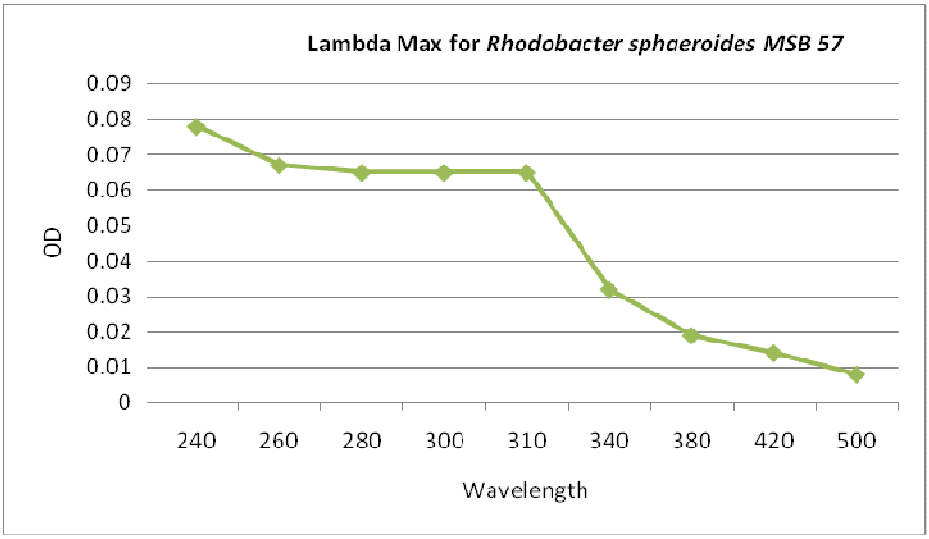



**Figure : 4 GC/MS ANALYSIS:**

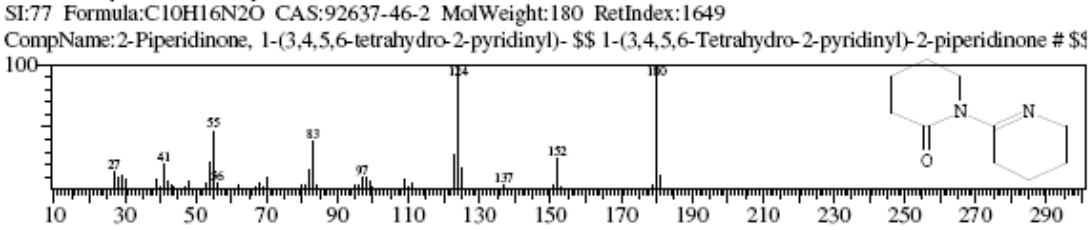
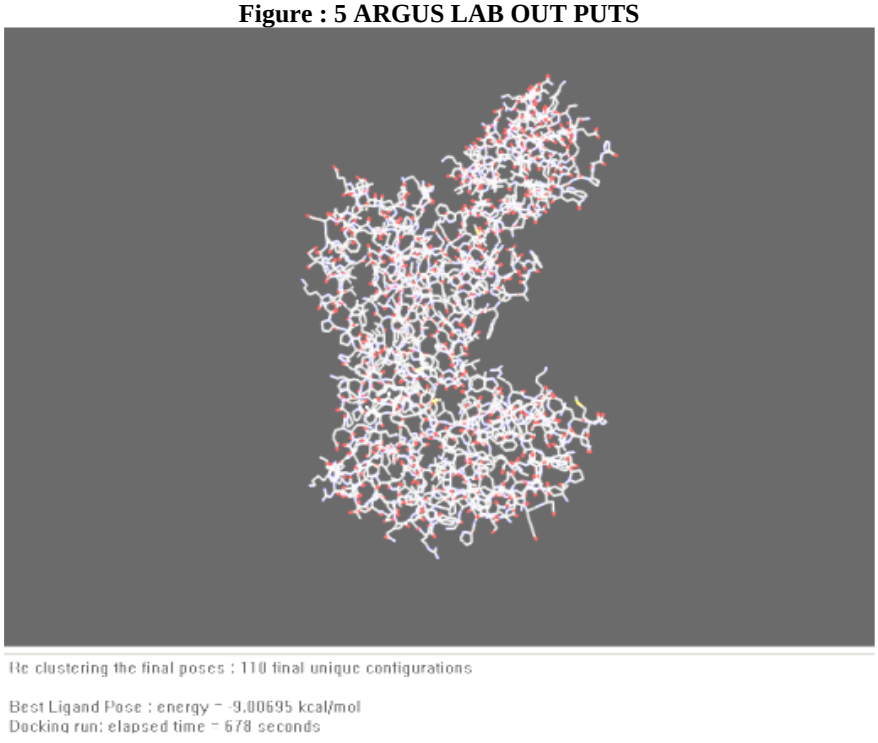
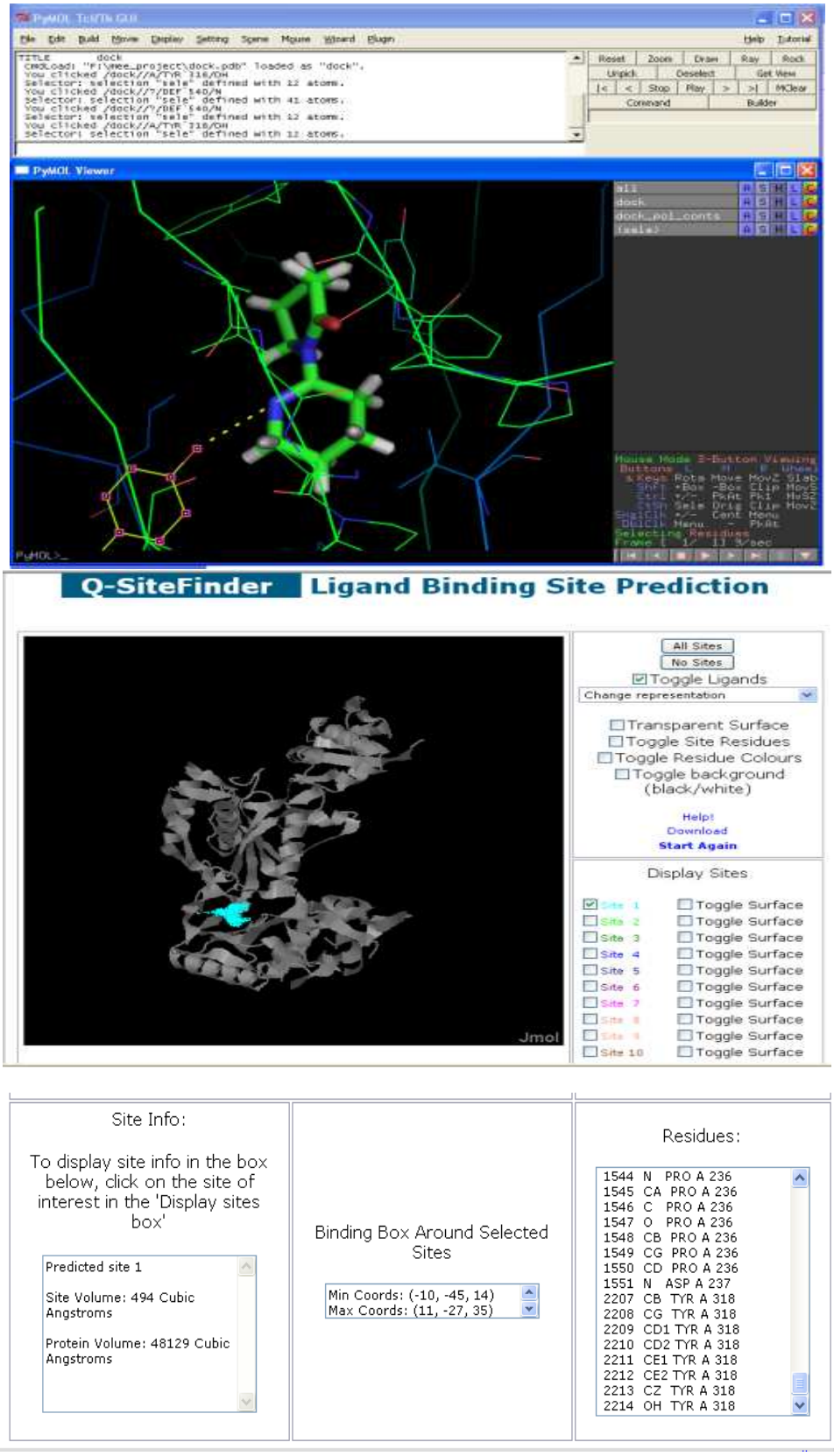



**Figure : 5 ARGUS LAB OUT PUTS**

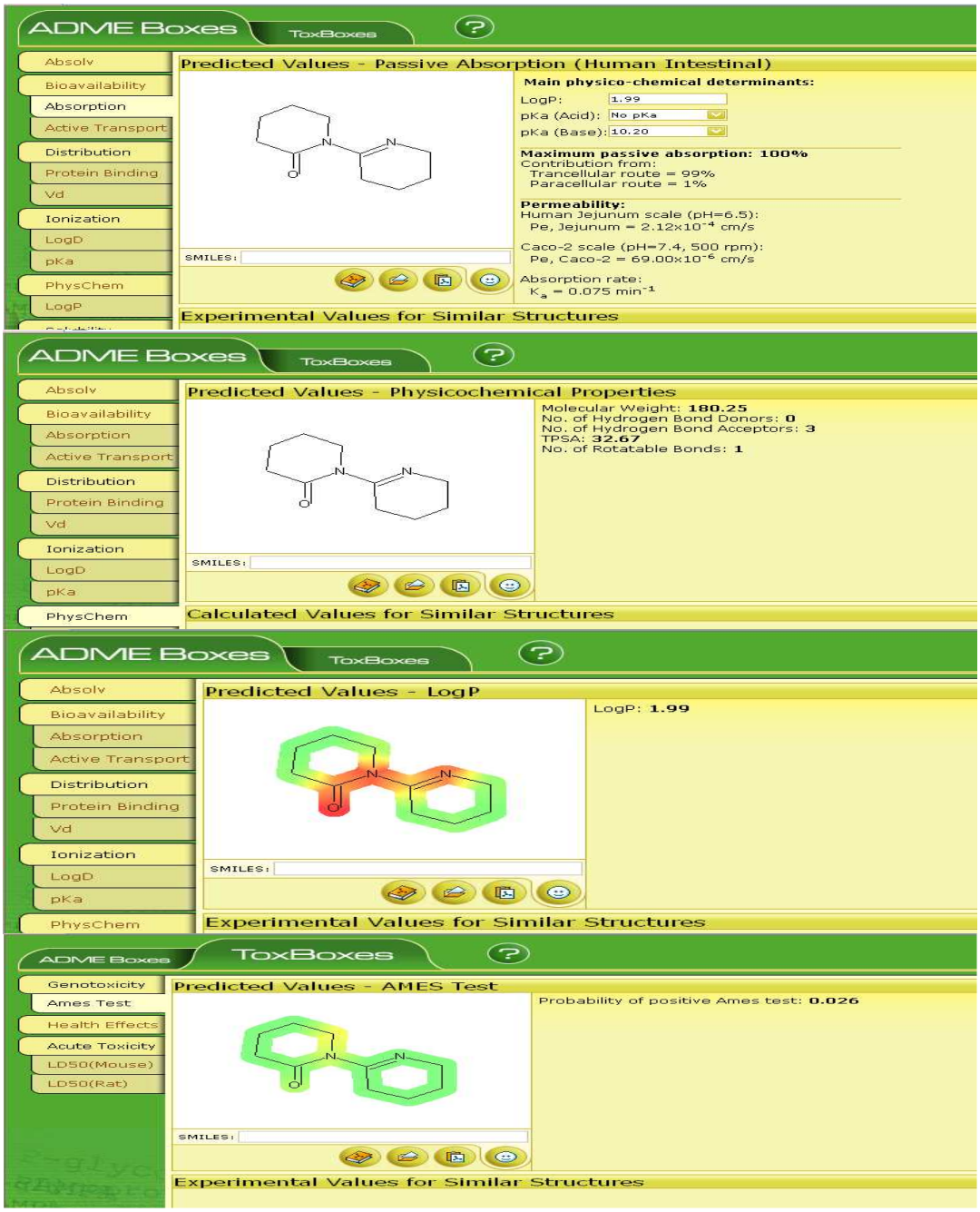



PyMOL Viewer
Q-SiteFinder
Ligand Binding Site Prediction
All Sites
No Sites
Toggle Ligands
Change representation
Transparent Surface
Toggle Site Residues
Toggle Residue Colours
Toggle background (black/white)
Help!
Download
Start Again
Display Sites
Site 1
Site 2
Site 3
Site 4
Site 5
Site 6
Site 7
Site 8
Site 9
Site 10
Toggle Surface
Jmol
Site Info:
To display site info in the box below, click on the site of interest in the 'Display sites box'
Predicted site 1
Site Volume: 494 Cubic Angstroms
Protein Volume: 48129 Cubic Angstroms
Binding Box Around Selected Sites
Min Coords: (-10, -45, 14)
Max Coords: (11, -27, 35)
Residues:
1544 N PRO A 236
1545 CA PRO A 236
1546 C PRO A 236
1547 O PRO A 236
1548 CB PRO A 236
1549 CG PRO A 236
1550 CD PRO A 236
1551 N ASP A 237
2207 CB TYR A 318
2208 CG TYR A 318
2209 CD1 TYR A 318
2210 CD2 TYR A 318
2211 CE1 TYR A 318
2212 CE2 TYR A 318
2213 CZ TYR A 318
2214 OH TYR A 318

**Reverse Transcriptase**

| RESIDUE | ATOM | LIGAND |
|---|---|---|
| TYR 318 | OH | N |

**Figure : 6 ADME/TOX RESULTS**



**SOLUBILITY TEST**

Solubility of the extracted compounds were studied using Water, 5% NaOH, 5% $NaHCO_3$, 5% HCl, Ethyl acetate, Chloroform, Methanol, Petroleum ether and Ethanol. Based on the result it was predicted as the compounds were partially polaric.

**THIN LAYER CHROMATOGRAPHY RESULTS:**

The qualitative TLC result revealed that there are different kinds of compound present in the crude ethyl acetate extract of both the test organisms because there were the presence of UV absorbing compounds, alkaloids and protein derivatives.

**LAMBDA MAX**

Lambda max was determined for the compounds. Wave length from 240-520 were selected. When the UV absorptive rate and visible absorptive rate was compared, there was 6 fold increase in the OD value in UV range( Figure : 3).

GC/MS analysis out puts were analyzed using NIST05 library search tool. Which revealed the presence of 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl) in the bioactive compound.

**TABLE : 1 ARGUS LAB OUT PUTS**

| S.NO | Name of the compounds | Name of the target | Source | Docking score | |
|---|---|---|---|---|---|
| 1. | 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl) | Revere Transcriptase | HIV | -9.00695 Kcal/mol | Residue-Tyr 318<br>Atom-OH<br>Ligand-N |

Michael W. Miller & Jack D. Scott, 2006, stated that the present invention of bi piperidinyl compounds as inhibitors of the CCR5 receptors, methods of preparing such compounds pharmaceutical composition containing one or more such compound, which has inhibition CCR5. The invention also relates to the use combination of a compound of this invension and one more antiviral agents are useful in the treatment of HIV.

As per the report given by Hauber & Ilona, 2009, a pharmaceutical composition comprising the composition of the invention and a pharmaceutically suitable excipient . In the context of the invention, a pharmaceutical composition (medicament) is useful for treatment or alleviation of a disease or disorder in humans and/or animals. The excipient may be a carrier. The pharmaceutical composition may be formulated, e.g., for oral or intravenous administration. The pharmaceutical composition may be liquid or solid or in gel form. In particular, water, ethanol and/or DMSO may be used as an excipient. In the context of the present invention, "a" is not to be construed as limited to "one", i.e., for example, more than one excipient may be used, or more than one compound of the invention may be used. Further active ingredients may be incorporated in the pharmaceutical composition or administrated in combination with it. For example, other antiviral agents, such as those mentioned above, may be combined with the pharmaceutical composition of the invention for an antiretro- viral medicament, or a tyrosine kinase inhibitor may be combined with the pharmaceutical composition of the invention for medicament for treatment of a proliferative disease. The present invention provides the use of a composition comprising a 4-oxo-piperidine-carboxylate according to Formula I.

In this present study also 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl)was used to study their AntiHIV activity. In the previous study, the compound was synthetically prepared but in this present study a natural compound similar to that which was produced by the bacteria *Rhodobacter sphaeroides MSB 57* had been used. The docking score for the compound was -9.00695 Kcal/mol. There was one hydrogen bond formation in-between the ligand's tyrosine, in the 318$^{th}$ position. ADME TOX result also revealed that it has the ability to act as drug.

**CONCLUSION**

In conclusion the compound 2-piperidinone,1-(3,4,5,6-tetrahydro-2-pyridinyl), produced by the marine sponge associated bacteria *Rhodobacter sphaeroides MSB* 57 could act as an novel anti retroviral drug to control retro viral diseases including HIV.

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