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# Isolation and X-ray crystal structure of 1,3,9 trimethyl purine derivative having cytotoxic activities from marine sponge *Amphimedon viridis* (Duchassaing & Michelotti,1864)

Chemical Science

**KEYWORDS:** Sponge, Purines, NMR & Mass spectrometry, Natural Products, X-ray crystallography, Cytotoxic activity, Anti-inflammatory activity.

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| **Srinu Meesala** | Department of Biology, Indian Institute of Science Education and Research, Dr.Homi Bhabha Road, Pune-411008, India. |
| **Chandraiah Godugu** | National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad-500037, India. |
| **Milind G. Watve** | Department of Biology, Indian Institute of Science Education and Research, Dr.Homi Bhabha Road, Pune-411008, India. |

## ABSTRACT

A new purine derivative 1,3,9 trimethyl uric acid (1) was isolated from the marine sponge Amphimedon viridis and structure were confirmed by X-ray crystallography data. Compound 1 exhibited in vitro cytotoxic activity against breast cancer (MCF-7), human neuroblastoma (SHSY5Y), human non-small cell lung cancer (A549) and human liver cancer (HEPG2) with IC50 value in the range of 1-3 μM and also showed promising anti-inflammatory effects in LPS induced murine macrophage J774 in vitro models in the concentration range of 100-1000 nM.

### Introduction

Marine organisms comprise over 80% of the world's plant and animal species and thus offer an enormous resource for the discovery of chemotherapeutic agents (Blunt et al., 2010; Hu et al., 2011; Jha et al 2004) Marine invertebrates are the richest source of new metabolites as reported in marine libraries (Blunt et al., 2016). Different classes of compounds like terpenoids, Alkaloids, aliphatic compounds, steroids, carbohydrates, amino acids, and peptides have been reported from marine sponges(Leal et al., 2012). Recent research on natural products focused on the methylated purine derivatives/ analogs from the marine sponges(Rosemeyer, 2004; Michel et al., 2006) has showed potential therapeutic agents to treat an impressively wide range of diseases (Sufrin et al., 2009; Marina et al., 2009; Tsuyoshi et al., 2010). In this paper , we describe the isolation , X-ray crystal data of natural purine derivative from the marine sponge.

### Experimental Methods

#### General experimental details

Optical rotations were determined on a Rudolph Research Analytical (AUTOPOL V) polarimeter at a wavelength of 589 nm (sodium D line) using a 1.0-decimeter cell with a total volume of 1.0mL. The UV spectra were measured on an Agilent technologies carry series UV-VIS spectrophotometer and Infrared spectra on Bruker ALPHA. All solvents were of analytical grade. Column chromatography was performed on Merck silica gel (120-200 mesh) and Sephadex LH-20 (Sigma-Aldrich Chemie GmbH). The $^{1}H$ and $^{13}C$ NMR spectra acquired at Bruker 400 MHz (Internal standard: TMS). The chemical shifts ( ) are reported in ppm and coupling constants (J) in Hz. The positive ion HR-ESI-MS spectra were recorded on a Mass Q-TOF-LC-MS spectrometer (Bruker Daltonics). Reverse phase $C_{18}$ column(10x250mm), Agilent HPLC.

#### Collection of sponge

The sponge *Amphimedon viridis (*Duchassaing & Michelotti, 1864) was collected from Mirkarwada (N 18°19.092' E 072°57.343'), Maharashtra, INDIA in Feb 2016. The sponge was identified by Dr. Satish S. Mokashe, Associate Professor. A voucher specimen (No. BEM 65) was deposited in Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Maharashtra, INDIA.

#### X-ray diffraction study of compound 1

#### Crystallization:

Purified compound five milligrams was dissolved in methanol and 1,4 dioxane(1:4) at room temperature and kept for slow evaporation in a vial closed with two layers of tissue paper. Clear white crystals were observed after full evaporation. Single crystal X-ray diffraction data were collected on an Oxford Xcalibur Eos (Mova) diffractometer at 100 K using MoK radiation (=0.7107Å) with X-ray generator operating at 50 kV and 1 mA(Agilent Technologies, 2011). A single crystal 0.3 X0.03 XO.55 mm size was used for X-ray diffraction study. Its structure was solved by direct methods using the SHELXS-97 program (Sheldrick 1997) and refined by full-matrix least squares on F2 using an SHELXL-97 program (Sheldrick, 1997).Data of atomic coordinates, bond distances and angles, anisotropic displacement parameters, hydrogen atom coordinates and torsion angles are deposited with the Cambridge Crystallographic Data Centre (http://www. ccdc.cam.ac.uk). CCDC 1500287.

#### Cytotoxic assay

Cytotoxicity was evaluated in against breast cancer (MCF-7), human neuroblastoma(SHSY5Y), human non-small cell lung cancer (A549) and human liver cancer (HEPG2) cell lines. Cell proliferation was followed by the colorimetric MTT test (Visconti et al., 1991).

#### Anti-Inflammatory activity

The in vitro anti-inflammatory effects are studied by using macrophages like J774 cell lines. The nitric oxide (NO) produced due to induction of iNOS is one of the key inflammatory marker used for measurement inflammation in in-vitro conditions. J774 cells were seeded in a 24-well cell culture plates at a density of $10^5$ cells/well. Cells were allowed to adhere to wells overnight at 37°C and 5% CO2. Then cells were treated with different concentration of compound **1** dissolved in DMSO. After 1h, cells were stimulated with lipopolysaccharide (LPS) at 1μg/ml. After 24h, cell culture supernatants were collected and used for NO estimation. NO estimation was done by Giustarini et al., 2008, briefly 50μL of the supernatant was incubated with 100μL of Griess reagent. After 10 min at room temperature, the absorbance was measured at 550 nm. Nitrite concentration was calculated using sodium nitrite as standard.

### Results and discussion

We have recently initiated a program in search of biologically active compounds from marine sponges. We collected *Amphimedon viridis* during field trip in Feb 2016 from Mikarwada, Arabian sea, INDIA. The freeze-dried sponge (0.86g)was extracted sequentially with hexane, chloroform, and methanol. These extracts were tested against cancer cell lines in preliminary studies. The cytotoxic activity was only found in the methanol extract which exhibited at 10 and 50 μg/ml, 10.2% and 23.82% inhibition respectively (supporting information figure S1). Hexane and DCM extracts were lacked cytotoxic activity. The MeOH extract(0.2g) was subjected to fractionation using gel permeation chromatography Sephadex LH-20 eluted with MeOH:$H_20$ 1:1, which resulted in 12(F1-F12) fractions. Two fractions F6 and F 7 showed strong in vitro cytotoxic activity on MCF-7 cells with 25.6% and 78.2 % inhibition at 10 and 50 μg/ml, respectively (supporting information figure S1). Fractions 6 and 7

together purified by $C_{18}$ semi-prep column, reversed-phase HPLC using 0.01% aqueous TFA in water: MeOH gradient to yield new compound **1** (5mg, 0.005 % dry weight, $t_R$= 21.6) (supporting information figure S2).

**Compound 1**

Compound **1** obtained as an amorphous white powder, the specific rotation was $[\alpha]^{25}_{D}$ = +2.8(c 1.0, $CH_3OH$). The ESI-MS-QTOF exhibited a pseudo molecular ion peak at m/z 211.084 $[M+H]^+$ (Calcd. 211.083) (supporting information figure S3) corresponding to the molecular formula of $C_8H_{10}N_4O_3$, indicating six degrees of unsaturation. The IR and UV (MeOH) spectra of compound **1** showed bands at 3323, 2945, 2833, 1658, 1450, 1414,1113 and 1018 $cm^{-1}$ and at $\lambda_{max}$ 210 and 293 nm, respectively(supporting information figure S4, S5). These UV and IR values were similar to 1,3 dimethyl isoguanine reported by Roberto G.S.Berlinck group.[14] The $^1$H-NMR spectrum of compound (**1**) was showing three methyl singlet's at $\delta_H$ 3.64, 3.49, 3.20 and one NH proton at $\delta_H$8.19 in DMSO -d6.(supporting information figure S6)

The $^{13}$C-NMR spectrum of **1** showed six quaternary carbons at $\delta_C$ 156.1,150.9,152.7,137.1,97.7 and three methyl carbons $\delta_C$ 28.3, 31.4, 29.87 (supporting information figure S7). The $^1$H, $^{13}$C data showed similarity with previously reported 1,3-Dimethylisoguanine (Scott et al., 1997; Chehade et al., 1997) and 1,3,7-trimethylguanine (Perry et al., 1987) but the presence of six quaternary carbons and mass confirmed that compound **1** was a new natural purine derivative from the marine sponge *Amphimedon viridis*.

Crystal system: Orthorhombic; space group *P*bca; *a* (Å) 6.7512(10), *b* (Å)= 16.043(2), *c* (Å)= 16.241(12). Volume (Å$^3$)= 1759.1(4), Z=8. The intensities were measured at temperature K 100(2), using CuK radiation and 13475 unique reflections collected. $R_{\_all}$ = 0.0551,$wR_2$= 0.1077. Full crystallographic details of compound **1** have been deposited in Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 1500287. The anisotropic displacement parameters, fractional atomic coordinates, torsion angles, bond angles and bond lengths of compound **1** is showed in supporting information table S1-S7, these values showed similarity with reported 8-oxopurine (Cimino et al.,1985). The compound **1** ORTEP representation and the atom-numbering scheme was showed in Figure 1 .The final structure of compound **1** was confirmed by X-ray analysis as 1,3,9 trimethyl uric acid, a methylated purine analog. To the best of our knowledge, this is the first report of compound **1** is another new natural purine derivative from the marine sponge *Amphimedon viridis.*

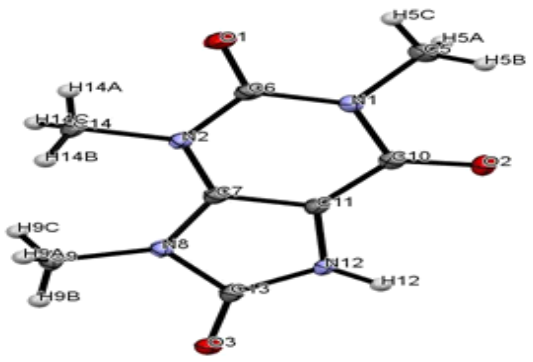


**Figure 1.** ORTEP diagram for compound 1

Compound 1 exhibited potent cytotoxic activity with IC50 value 1-3 µM against breast cancer (MCF-7), human neuroblastoma(SHSY5Y), human non-small cell lung cancer (A549) and human liver cancer (HEPG2) cell lines as showed in Figure 2& 3. Additionally, compound 1 also showed promising anti-inflammatory effects in LPS induced murine macrophage J774 in vitro models was showed in Figure 4. The LPS induced NO production was found to be significantly decreased in the concentration range of 100-1000 nM in the treated cells.

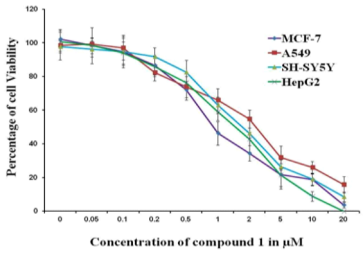


**Figure 2:** In vitro anticancer effects- The concentration dependent cytotoxic effect of compound 1 studied on different types of cell lines. Each data point is represented as mean±SEM (n=6-8) and data is represents the replicate of three independent experiments.

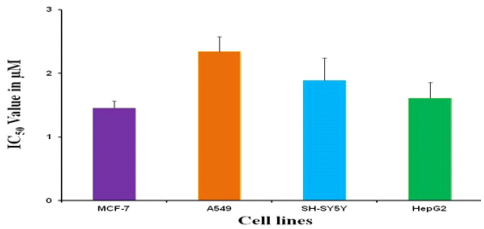


**Figure 3:** IC50 values for compound 1 on various cancer cell types. Each value is represented as mean±SEM (n=6-8) three independent experiments were performed.

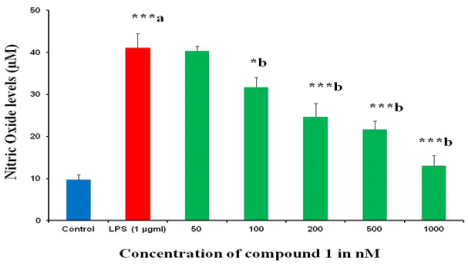


**Figure 4 :** Effects of compound 1 on NO production in J774 macrophages. Cells were stimulated with lipopolysaccharide (LPS) and effect of compound 1 on Nitric Oxide levels were studied. After 24 h incubation, NO released into the cell culture medium was estimated by Griess method. Each data point was represented as mean±SEM (n = 4-6). *p<0.05, ***P <0.001 a Vs control group and b Vs LPS alone group.

**Conclusion:**

In summary, this paper describes the isolation, bioactive guided purification and structure elucidation of a methylated purine derivative 1,3,9 trimethyl uric acid from the marine sponge Amphimedon viridis (Duchassaing & Michelotti, 1864), Mikarawadi, India. The compound exhibited potent in vitro cytotoxic activity as well as anti-inflammatory activity, which added further evidence that the sponge Amphimedon viridis (Duchassaing & Michelotti, 1864) is an abundant source of chemically diverse and biologically active secondary metabolites.

**Acknowledgments**

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Supplementary information

Supplementary data associated with this article can be found, in the online version.

The authors declare no competing financial interests.

**SUPPORTING INFORMATION**

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Isolation and X-ray crystal structure of 1,3,9 trimethyl purine derivative having cytotoxic activities from marine sponge Amphimedon viridis (Duchassaing & Michelotti,1864)

Srinu Meesala[a],* Chandraiah Godugu[b], Milind G Watve[a]
[a] Department of Biology, Indian Institute of Science Education and Research, Dr.Homi Bhabha Road, Pune-411008, India.

[b] National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad-500037, India.



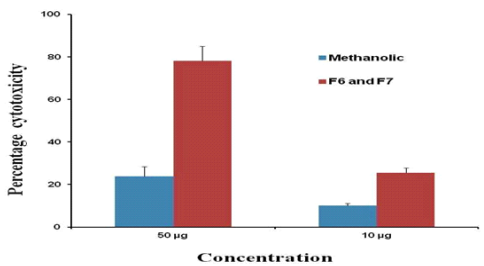


**Figure S1:** Cytotoxic effects of Methanol extract and fractions 6&7

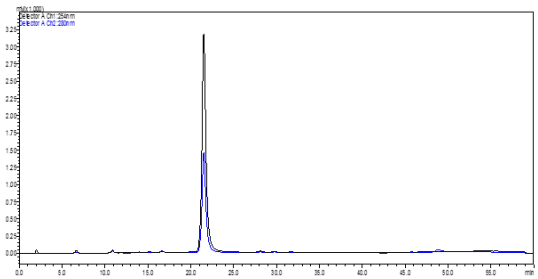

**Figure S2:** HPLC trace of fractions 6&7 were separated by using semi prep C18 column, compound eluted at tR 21.6.

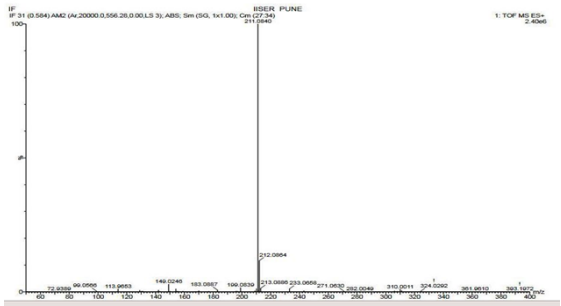

**Figure S3:** High resolution Mass spectra of compound 1

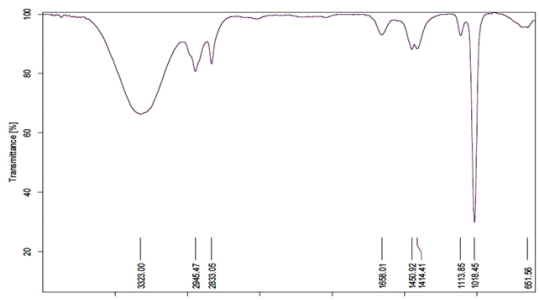


**Figure S4.** IR spectra of compound 1

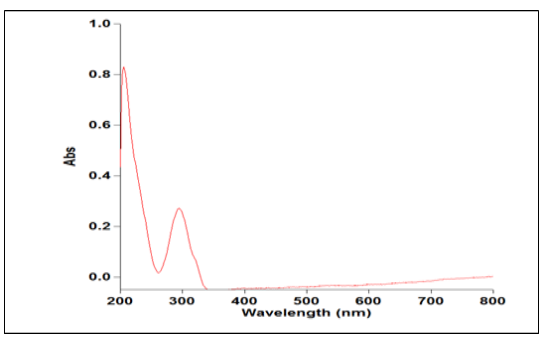


**Figure S5:** UV-Vis spectra of compound 1

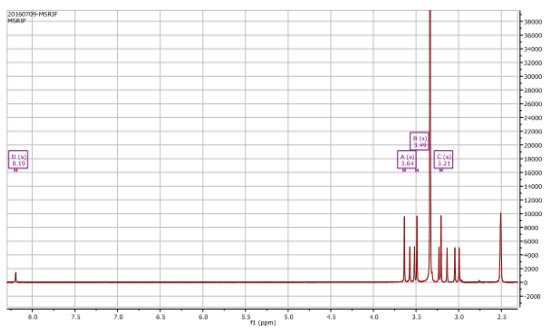

**Figure S6:** 1H NMR (400 MHz, DMSO-d6) spectra of compound 1

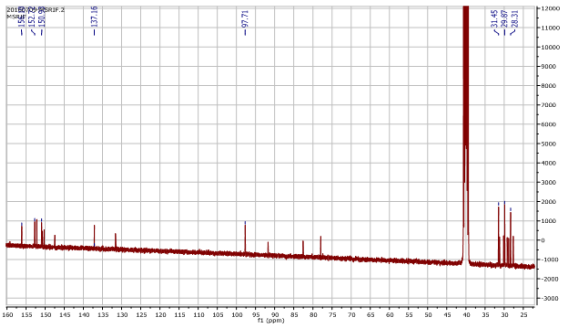

**Figure S7:** 13C NMR (400 MHz, DMSO-d6) spectra of compound 1

| | |
|---|---|
| Empirical formula | C8H10N4O3 |
| Formula weight | 210.20 |
| Temperature/K | 293(2) |
| Crystal system | orthorhombic |
| Space group | Pbca |
| a/Å | 6.7512(10) |
| b/Å | 16.043(2) |
| c/Å | 16.241(2) |
| α/° | 90 |
| β/° | 90 |
| γ/° | 90 |
| Volume/Å3 | 1759.1(4) |
| Z | 8 |
| ρcalcmg/mm3 | 1.587 |
| m/mm-1 | 0.125 |
| F(000) | 880.0 |
| Crystal size/mm3 | 0.3 X0.03 XO.55 mm |
| 2Θ range for data collection | 5.016 to 55.616° |
| Index ranges | -8 ≤ h ≤ 8, -20 ≤ k ≤ 20, -21 ≤ l ≤ 21 |
| Reflections collected | 13475 |
| Independent reflections | 2024[R(int) = 0.0498] |
| Data/restraints/parameters | 2024/0/139 |
| Goodness-of-fit on F2 | 1.047 |
| Final R indexes [I>=2σ (I)] | R1 = 0.0395, wR2 = 0.0980 |
| Final R indexes [all data] | R1 = 0.0551, wR2 = 0.1077 |
| Largest diff. peak/hole / e Å-3 | 0.21/-0.35 |

**Table S1:** Crystal data and structure refinement for compound 1

| Atom | x | y | z | U(eq) |
|---|---|---|---|---|
| O1 | -3819.8(16) | 1334.3(7) | 2059.6(6) | 20.1(3) |
| O2 | -438.3(16) | -952.6(7) | 1118.3(6) | 18.7(3) |
| O3 | 5275.9(15) | 1127.8(6) | 86.3(6) | 16.4(3) |
| N1 | -2141.0(18) | 201.5(8) | 1566.1(7) | 14.6(3) |
| N2 | -914.3(18) | 1572.3(8) | 1385.5(7) | 13.8(3) |
| N8 | 2409.6(18) | 1609.3(8) | 738.3(7) | 13.6(3) |
| N12 | 2724.5(18) | 256.7(8) | 521.7(7) | 14.4(3) |
| C5 | -3686(2) | -354(1) | 1887.1(9) | 19.2(3) |
| C6 | -2376(2) | 1054.2(10) | 1696.7(8) | 14.7(3) |
| C7 | 721(2) | 1226.9(9) | 1030.6(8) | 12.8(3) |
| C9 | 3139(2) | 2464.7(9) | 828.9(9) | 17.2(3) |
| C10 | -518(2) | -187.4(9) | 1177.0(8) | 14.0(3) |
| C11 | 903(2) | 392.7(9) | 903.8(8) | 13.6(3) |
| C13 | 3643(2) | 993.3(9) | 412.4(8) | 13.4(3) |
| C14 | -1129(2) | 2468.8(9) | 1534.9(9) | 18.4(3) |

**Table S2:** Fractional Atomic Coordinates (×104) and Equivalent Isotropic Displacement Parameters (Å2×103) for . Ueq is defined as 1/3 of of the trace of the orthogonalised UIJ tensor.

| Atom | $U^{11}$ | $U^{22}$ | $U^{33}$ | $U^{23}$ | $U^{13}$ | $U^{12}$ |
|---|---|---|---|---|---|---|
| O1 | 20.8(6) | 18.1(6) | 21.5(5) | -0.7(4) | 6.3(4) | 5.2(5) |
| O2 | 21.5(6) | 10.1(6) | 24.5(5) | -0.6(4) | 1.9(4) | 0.4(4) |
| O3 | 15.1(5) | 12.6(5) | 21.6(5) | 0.1(4) | 4.3(4) | 0.1(4) |
| N1 | 15.9(7) | 11.3(6) | 16.6(6) | 0.5(5) | 2.2(5) | 0.0(5) |
| N2 | 15.9(6) | 10.2(6) | 15.2(6) | -1.3(5) | 0.5(5) | 2.3(5) |
| N8 | 14.7(6) | 8.9(6) | 17.3(6) | -0.3(5) | 0.0(5) | 0.7(5) |
| N12 | 15.6(6) | 8.9(6) | 18.7(6) | -1.8(5) | 2.7(5) | 2.0(5) |
| C5 | 19.2(8) | 16.9(8) | 21.5(7) | 0.4(6) | 4.1(6) | -2.6(6) |
| C6 | 18.3(8) | 14.1(8) | 11.8(6) | 0.0(5) | -0.1(6) | 2.2(6) |
| C7 | 15.1(7) | 11.3(7) | 11.9(6) | 0.2(5) | -0.5(5) | 1.4(6) |
| C9 | 18.7(8) | 9.2(7) | 23.5(7) | -1.0(6) | 0.2(6) | -0.7(6) |
| C10 | 17.1(8) | 12.7(8) | 12.3(6) | 0.2(5) | -1.0(6) | 1.5(6) |
| C11 | 15.1(7) | 11.2(7) | 14.6(6) | -1.1(5) | 1.0(5) | 1.8(6) |
| C13 | 15.9(8) | 10.4(7) | 13.9(6) | 0.4(5) | -1.6(5) | 1.8(6) |
| C14 | 21.0(8) | 10.8(7) | 23.4(7) | -1.6(6) | 2.5(6) | 4.6(6) |

**Table S 3:** Anisotropic Displacement Parameters (Å2×103) for . The Anisotropic displacement factor exponent takes the form: -2π2[h2a*2U11+...+2hka×b×U12].

| Atom | Atom | Length/Å |
|---|---|---|
| O1 | C6 | 1.2244(18) |
| O2 | C10 | 1.2325(18) |
| O3 | C13 | 1.2420(17) |
| N1 | C5 | 1.4678(19) |
| N1 | C6 | 1.3934(19) |
| N1 | C10 | 1.4102(18) |
| N2 | C6 | 1.3859(19) |
| N2 | C7 | 1.3634(18) |
| N2 | C14 | 1.4657(19) |
| N8 | C7 | 1.3787(19) |
| N8 | C9 | 1.4654(19) |
| N8 | C13 | 1.3965(18) |
| N12 | C11 | 1.3949(18) |
| N12 | C13 | 1.3463(19) |
| C7 | C11 | 1.360(2) |
| C10 | C11 | 1.408(2) |

**Table S 4:** Bond lengths for compound 1

| Atom | Atom | Atom | Angle/° | Atom | Atom | Atom | Angle/° |
|---|---|---|---|---|---|---|---|
| C6 | N1 | C5 | 117.45(12) | N2 | C7 | N8 | 129.37(14) |
| C6 | N1 | C10 | 126.24(12) | C11 | C7 | N2 | 122.48(13) |
| C10 | N1 | C5 | 116.27(12) | C11 | C7 | N8 | 108.15(12) |
| C6 | N2 | C14 | 117.24(12) | O2 | C10 | N1 | 120.63(13) |
| C7 | N2 | C6 | 119.16(12) | O2 | C10 | C11 | 127.15(13) |
| C7 | N2 | C14 | 123.29(13) | C11 | C10 | N1 | 112.20(13) |
| C7 | N8 | C9 | 131.29(12) | N12 | C11 | C10 | 129.60(13) |
| C7 | N8 | C13 | 107.97(12) | C7 | C11 | N12 | 107.50(13) |
| C13 | N8 | C9 | 120.03(12) | C7 | C11 | C10 | 122.79(13) |
| C13 | N12 | C11 | 109.12(12) | O3 | C13 | N8 | 124.61(13) |
| O1 | C6 | N1 | 121.62(14) | O3 | C13 | N12 | 128.14(14) |
| O1 | C6 | N2 | 121.49(14) | N12 | C13 | N8 | 107.25(12) |
| N2 | C6 | N1 | 116.88(12) | | | | |

**Table S 4:** Bond lengths for compound 1

| Atom | Atom | Atom | Angle/° | Atom | Atom | Atom | Angle/° |
|---|---|---|---|---|---|---|---|
| C6 | N1 | C5 | 117.45(12) | N2 | C7 | N8 | 129.37(14) |
| C6 | N1 | C10 | 126.24(12) | C11 | C7 | N2 | 122.48(13) |
| C10 | N1 | C5 | 116.27(12) | C11 | C7 | N8 | 108.15(12) |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C6 | N2 | C14 | 117.24(12) | O2 | C10 | N1 | 120.63(13) |
| C7 | N2 | C6 | 119.16(12) | O2 | C10 | C11 | 127.15(13) |
| C7 | N2 | C14 | 123.29(13) | C11 | C10 | N1 | 112.20(13) |
| C7 | N8 | C9 | 131.29(12) | N12 | C11 | C10 | 129.60(13) |
| C7 | N8 | C13 | 107.97(12) | C7 | C11 | N12 | 107.50(13) |
| C13 | N8 | C9 | 120.03(12) | C7 | C11 | C10 | 122.79(13) |
| C13 | N12 | C11 | 109.12(12) | O3 | C13 | N8 | 124.61(13) |
| O1 | C6 | N1 | 121.62(14) | O3 | C13 | N12 | 128.14(14) |
| O1 | C6 | N2 | 121.49(14) | N12 | C13 | N8 | 107.25(12) |
| N2 | C6 | N1 | 116.88(12) | | | | |

**Table S5:** Bond Angles for compound 1

| A | B | C | D | Angle/° | A | B | C | D | Angle/° |
|---|---|---|---|---|---|---|---|---|---|
| O2 | C10 | C11 | N12 | -1.2(2) | C7 | N8 | C13 | O3 | 178.87(12) |
| O2 | C10 | C11 | C7 | -176.91(14) | C7 | N8 | C13 | N12 | -1.07(15) |
| N1 | C10 | C11 | N12 | 177.51(13) | C9 | N8 | C7 | N2 | 11.3(2) |
| N1 | C10 | C11 | C7 | 1.81(19) | C9 | N8 | C7 | C11 | -169.19(14) |
| N2 | C7 | C11 | N12 | 179.10(12) | C9 | N8 | C13 | O3 | -9.7(2) |
| N2 | C7 | C11 | C10 | -4.4(2) | C9 | N8 | C13 | N12 | 170.36(12) |
| N8 | C7 | C11 | N12 | -0.43(15) | C10 | N1 | C6 | O1 | -178.20(12) |
| N8 | C7 | C11 | C10 | 176.10(12) | C10 | N1 | C6 | N2 | 2.8(2) |
| C5 | N1 | C6 | O1 | -0.50(19) | C11 | N12 | C13 | O3 | -179.13(13) |
| C5 | N1 | C6 | N2 | -179.55(12) | C11 | N12 | C13 | N8 | 0.81(15) |
| C5 | N1 | C10 | O2 | -0.05(19) | C13 | N8 | C7 | N2 | -178.56(13) |
| C5 | N1 | C10 | C11 | -178.86(11) | C13 | N8 | C7 | C11 | 0.93(15) |
| C6 | N1 | C10 | O2 | 177.68(13) | C13 | N12 | C11 | C7 | -0.25(15) |
| C6 | N1 | C10 | C11 | -1.14(19) | C13 | N12 | C11 | C10 | -176.46(14) |
| C6 | N2 | C7 | N8 | -174.68(13) | C14 | N2 | C6 | O1 | 2.19(19) |
| C6 | N2 | C7 | C11 | 5.9(2) | C14 | N2 | C6 | N1 | -178.76(12) |
| C7 | N2 | C6 | O1 | 176.05(12) | C14 | N2 | C7 | N8 | -1.2(2) |
| C7 | N2 | C6 | N1 | -4.90(18) | C14 | N2 | C7 | C11 | 179.36(13) |

**Table S6:** Torsion Angles for compound 1

| Atom | x | y | z | U(eq) |
|---|---|---|---|---|
| H12 | 3187 | -221 | 379 | 17 |
| H5A | -3186 | -651 | 2356 | 29 |
| H5B | -4065 | -744 | 1468 | 29 |
| H5C | -4818 | -30 | 2047 | 29 |
| H9A | 2341 | 2834 | 502 | 26 |
| H9B | 4490 | 2493 | 647 | 26 |
| H9C | 3063 | 2628 | 1397 | 26 |
| H14A | -2497 | 2597 | 1634 | 28 |
| H14B | -674 | 2772 | 1062 | 28 |
| H14C | -356 | 2624 | 2007 | 28 |

**Table S7:** Hydrogen Atom Coordinates (Å×104) and Isotropic Displacement Parameters (Å2×103)

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