SUPPORTING INFORMATION

DIRECT PHOSPHORYLATION OF PSILOCIN ENABLES OPTIMIZED cGMP KILOGRAM-SCALE MANUFACTURE OF PSILOCYBIN

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I. Certificate of Analysis

Chemical Formula: C₁₂H₁₇N₂O₄P
Monoisotopic Mass: 284.0926

| Test                              | Specification                      | Result                                                                 |
|----------------------------------|------------------------------------|------------------------------------------------------------------------|
| Appearance                       | Report result                      | Off-white solid                                                        |
| Identification by FTIR (ATR)     | Concordant with structure           | Concordant with structure                                               |
| Identification by ¹H NMR (D₂O)   | Conforms to reference spectrum     | Conforms to reference spectrum                                         |
| Identification by MS             | Concordant with structure           | Concordant with structure                                               |
| Identification by UPLC           | Retention time consistent with     | Retention time consistent with reference standard                      |
|                                  | reference standard                 |                                                                        |
| Assay by UPLC                    | 97-102% w/w (anhydrous)            | 99.7% w/w (anhydrous)                                                 |

| Impurities by UPLC               | Psilocin (metabolite impurity)     | Psilocin (metabolite impurity)                                         |
|                                  | NMT 1.0% area                      | < 0.05% area                                                           |
|                                  | Individual identified impurities   | Individual identified impurities                                       |
|                                  | NMT 0.5% area                      | 0.12% area                                                             |
|                                  | Any unidentified impurity          | Any unidentified impurity                                              |
|                                  | NMT 0.10% area                     | < 0.05% area                                                           |
|                                  | Total unknown impurity content     | Total unknown impurity content                                         |
|                                  | NMT 2.0% area                      | 0.10% area                                                             |
|                                  | Report all individual impurities   | Report all individual impurities                                       |
|                                  | ≥ 0.05% area                       |                                                                        |

| Water Content by KF              | ≤ 2% w/w                           | 0.49% w/w                                                              |

| Residual Solvents by GC          | Acetone NMT 5000 ppm               | Acetone < 1244 ppm (LOQ)                                              |
|                                  | DCM NMT 600 ppm                    | DCM < 153 ppm (LOQ)                                                   |
|                                  | DIPE NMT 5000 ppm                  | DIPE < 1247 ppm (LOQ)                                                |
|                                  | IPA NMT 5000 ppm                   | IPA < 1246 ppm (LOQ)                                                 |
|                                  | MeOH NMT 3000 ppm                  | MeOH < 747 ppm (LOQ)                                                 |
|                                  | Me-THF NMT 720 ppm                 | Me-THF < 180 ppm (LOQ)                                               |
|                                  | n-Heptane NMT 5000 ppm             | n-Heptane < 1246 ppm (LOQ)                                           |
|                                  | THF NMT 720 ppm                    | THF < 182 ppm (LOQ)                                                  |
|                                  | Triethylamine NMT 5000 ppm         | Triethylamine < 1247 ppm (LOQ)                                       |

| Elemental Impurities by ICP-MS   | As NMT 1.5 ppm                     | As < 0.605 ppm (LOQ)                                                 |
|                                  | Cd NMT 0.5 ppm                     | Cd < 0.200 ppm (LOQ)                                                 |
|                                  | Pb NMT 0.5 ppm                     | Pb < 0.197 ppm (LOQ)                                                 |
|                                  | Hg NMT 3 ppm                       | Hg < 1.20 ppm (LOQ)                                                  |
|                                  | Co NMT 5 ppm                       | Co < 2.00 ppm (LOQ)                                                  |
|                                  | V NMT 10 ppm                       | V < 4.04 ppm (LOQ)                                                   |
|                                  | Ni NMT 20 ppm                      | Ni < 7.99 ppm (LOQ)                                                  |
|                                  | Pd NMT 10 ppm                      | Pd < 4.00 ppm (LOQ)                                                  |
|                                  | Li NMT 100 ppm                     | Li < 39.7 ppm (LOQ)                                                  |

| Polymorph by XRPD               | Conforms to reference diffractogram | Conforms to reference diffractogram                                  |
| Residue on Ignition             | ≤ 0.5% w/w                          | 0.29% w/w                                                            |

Table S1: Psilocybin API Certificate of Analysis.
II. Estimated Solubility of Psilocybin

The solubility of psilocybin was estimated using an aliquot addition method. Aliquots of test solvent were added to an accurately weighed sample (~20 mg) of psilocybin at ambient temperature, agitated between aliquot additions, and volume recorded when all solids dissolved. The aliquot volumes were typically 50-1000 μL. Complete dissolution of the test material was determined by visual inspection. If dissolution did not occur after the last aliquot of solvent was added (typically ~40 volumes of solvent), the sample was subjected to two cycles of the following temperature cycling protocol on a Clarity crystallization station:

1. Heat from 20 °C to within 3 °C of solvent boiling point (or 100 °C, whichever was lower) at 0.5 °C/minute.
2. Cool to 20 °C at 0.2 °C/minute.
3. Stirrer speed 300 rpm.

Samples were then held at ambient temperature for 18 hours to maximize the chance of precipitation. The solubility values for psilocybin were expressed as a range and rounded to the nearest whole number (Table S2). From this data, solvent systems were grouped in the following manner (see Table S3):

1. **Solvents**: Psilocybin was soluble in ≤ 40 volumes (≥ 25 mg/mL) at ambient temperature.
2. **Soluble with heating**: Psilocybin was not soluble in 40 volumes at ambient temperature but dissolved at higher temperatures. These solvents could be regarded as possible solvents for cooling crystallizations.
3. **Anti-solvents**: Psilocybin was not soluble in 40 volumes at all temperatures studied.

The solubility was estimated from these experiments based on the total solvent used to provide complete dissolution. It should be noted that the actual solubility may be greater than that calculated because of the use of solvent aliquots that were too large or due to a slow rate of dissolution. The material was insoluble in most of the solvents tested but exhibited the highest solubility in TFE.

| Solvent                  | Solubility Range (mg/mL) | \( T_{\text{diss}} \) (°C) | \( T_{\text{cryst}} \) (°C) |
|--------------------------|--------------------------|-----------------------------|-----------------------------|
| Acetone                  | < 21                     | -                           | -                           |
| Acetonitrile             | < 21                     | -                           | -                           |
| Dichloromethane          | < 20                     | -                           | -                           |
| Diethyl ether            | < 20                     | -                           | -                           |
| Dimethyl formamide       | < 20                     | -                           | -                           |
| Dimethyl sulfoxide       | < 20                     | -                           | -                           |
| Ethyl acetate            | < 21                     | -                           | -                           |
| Methanol                 | < 21                     | -                           | -                           |
| Methyl ethyl ketone      | < 20                     | -                           | -                           |
| N-methyl pyrrolidone     | < 20                     | -                           | -                           |
| Tetrahydrofuran          | < 20                     | -                           | -                           |
| Toluene                  | < 20                     | -                           | -                           |
| Trifluoroethanol         | 136-204                  | -                           | -                           |
| Water                    | < 20                     | -                           | -                           |
| 50:50 DMSO/Water         | < 11                     | -                           | -                           |
| 25:75 DMSO/Water         | < 10                     | -                           | -                           |
| 75:25 Water/DMSO         | 20-23                    | -                           | -                           |
| 50:50 Methanol/Water     | < 10                     | 32                          | -                           |
| 50:50 THF/Water          | < 11                     | 28.8                        | -                           |
| 50:50 Acetone/Water      | < 11                     | 45.5                        | 28                          |
| 50:50 TFE/Methanol       | 15-16                    | -                           | -                           |
| Solvents                  | Soluble with Heating       | Anti-Solvents         |
|---------------------------|----------------------------|-----------------------|
| 50:50 TFE/Acetone         | < 10                       | -                     |
| 50:50 TFE/Water           | < 10                       | -                     |
| 50:50 TFE/THF             | < 11                       | -                     |
| 25:75 Water/Methanol      | < 10                       | -                     |
| 25:75 Water/THF           | < 11                       | -                     |
| 25:75 Water/Acetone       | < 10                       | -                     |
| 25:75 Water/TFE           | < 10                       | -                     |
| 75:25 Water/TFE           | < 11                       | -                     |

Table S2: Solubility estimates of psilocybin at 20 °C.

| Solvents                  | Soluble with Heating       | Anti-Solvents         |
|---------------------------|----------------------------|-----------------------|
| Trifluoroethanol          | 50:50 Methanol/Water       | Acetone               |
| -                         | 50:50 THF/Water            | Acetonitrile          |
| -                         | 50:50 Acetone/Water        | Dichloromethane       |
| -                         | -                          | Diethyl ether         |
| -                         | -                          | Dimethyl formamide    |
| -                         | -                          | Dimethyl sulfoxide    |
| -                         | -                          | Ethyl acetate         |
| -                         | -                          | Methanol              |
| -                         | -                          | Methyl ethyl ketone   |
| -                         | -                          | N-methyl pyrrolidone  |
| -                         | -                          | Tetrahydrofuran       |
| -                         | -                          | Toluene               |
| -                         | -                          | Water                 |
| -                         | -                          | 50:50 DMSO/Water      |
| -                         | -                          | 25:75 DMSO/Water      |
| -                         | -                          | 75:25 Water/DMSO      |
| -                         | -                          | 50:50 TFE/Methanol    |
| -                         | -                          | 50:50 TFE/Acetone     |
| -                         | -                          | 50:50 TFE/Water       |
| -                         | -                          | 50:50 TFE/THF         |
| -                         | -                          | 25:75 Water/Methanol  |
| -                         | -                          | 25:75 Water/THF       |
| -                         | -                          | 25:75 Water/Acetone   |
| -                         | -                          | 25:75 Water/TFE       |
| -                         | -                          | 75:25 Water/TFE       |

Table S3: Solvent systems grouped into categories.
III. XRD Diffractograms

XRPD analyses were performed using a Panalytical Xpert Pro diffractometer equipped with a Cu X-ray tube and a Pixcel detector system. The samples were analyzed at ambient temperature in transmission mode and held between low density polyethylene films. Diffraction data were collected in the 2θ range of 3-40° with a step size of 0.013° and a counting time of 2.2 seconds or ~5 min run time. XRPD patterns were sorted and manipulated using HighScore Plus 2.2c software.

![Figure S1: XRPD pattern of psilocybin Form A anhydrate.](image1)

![Figure S2: XRPD pattern of psilocybin Form B trihydrate.](image2)
IV. UPLC Methodology and Chromatograms

UPLC analyses (identification, assay, and impurity content) were carried out on an Agilent 1290 system with an Infinity Diode Array Detector. The chromatographic separation was performed using a YMC-Triart C18 metal free column (150 x 2.1 mm, 1.9 µm) at 30 °C. The mobile phases consisted of 0.1% phosphoric acid in water and 0.1% phosphoric acid in methanol. The mobile phase was delivered at a flow rate of 0.3 mL/min in gradient mode over a total run time of 30 minutes. Identification of psilocybin was performed by injecting 2 µL volume of 0.1 mg/mL sample with the DAD set to 220 nm.

| Time (minutes) | Mobile Phase A (%) | Mobile Phase B % |
|----------------|-------------------|------------------|
| 0              | 95                | 5                |
| 10             | 95                | 5                |
| 12             | 85                | 15               |
| 17             | 85                | 15               |
| 23             | 70                | 30               |
| 25             | 95                | 5                |
| 30             | 95                | 5                |

Table S4: UPLC Gradient conditions.

| Name                  | Retention Time (minutes) | Relative Retention Time | RRF  | Correction Factor (CF) |
|-----------------------|--------------------------|-------------------------|------|------------------------|
| Pyrophosphate psilocybin | 3.7                      | 0.58                    | N/A  | N/A                    |
| Psilocybin            | 6.4                      | N/A                     | N/A  | N/A                    |
| Psilocin              | 14.2                     | 2.21                    | 1.49 | 0.67                   |

Table S5: Typical retention times, relative retention times, and RRF/CF values for identified impurities.

Figure S3: Typical blank diluent injection (20 mM potassium phosphate buffer pH 2.0) chromatogram output.
Figure S4: Typical standard/sample chromatogram.

Figure S5: Typical standard/sample chromatogram (expanded).
V. Isolation and Characterization of Pyrophosphate Impurity

Background

Work was performed to elucidate the structure of an unknown impurity at RRT 0.6 present at a level of approximately 0.10% in GMP manufactured psilocybin. Crude psilocybin was isolated as a precipitate from purified water and was purified by repeated slurries in methanol and in water to a target specification of not less than (NLT) 98.0 % and no single unknown impurity greater than 0.09%. Isolated purity of both the wet cake and dried psilocybin indicated a purity in excess of 99% but with a single largest unknown impurity at a level of ca. 0.10%. The retrospective impurity (RRT 0.60 UPLC) was predominantly formed in the quench sequence of the synthesis work-up at levels of typically up to 7%. Upon crude isolation the level typically falls to < 1% and subsequent successive slurry treatments in methanol and water purge the level to < 0.15%. Thus, an attempt was made to either enrich or isolate this impurity to allow for potential structural elucidation.

Enrichment and isolation by reversed-phase preparative chromatography - During the purification sequence of the GMP manufactured psilocybin a series of slurries were performed, initially in methanol and then purified water. Analysis of the filtrate streams from these sequences indicated that the impurity at RRT 0.60 was enriched in the methanol slurry filtrate as indicated in Figure S6 below.

![Figure S6: Chromatogram of RRT 0.60 enriched MeOH filtrate from psilocybin GMP manufacture.](image)

| Name   | Retention Time | Area     | % Area | Height |
|--------|----------------|----------|--------|--------|
| 1      | 2.909          | 4090     | 0.18   | 665    |
| 2      | Impurity       | 3.409    | 188511 | 8.48   | 40920  |
| 3      | Psilocybin     | 5.649    | 1699104| 76.46  | 244225 |
| 4      | 6.390          | 6703     | 0.30   | 368    |
| 5      | Psilocin       | 13.507   | 252045 | 11.34  | 29993  |
| 6      | 14.062         | 5782     | 0.26   | 665    |
| 7      | 14.226         | 7134     | 0.32   | 369    |
| 8      | 14.755         | 355      | 0.02   | 68     |
| 9      | 15.305         | 2903     | 0.13   | 457    |
| 10     | 16.170         | 28338    | 1.28   | 3381   |
| 11     | 16.862         | 17074    | 0.77   | 2229   |
| 12     | 17.054         | 9201     | 0.41   | 1062   |
| 13     | 17.421         | 974      | 0.04   | 77     |

Table S6: Chromatographic data for detected peaks upon injection of enriched MeOH filtrate.
A sample of the filtrate was loaded onto a reversed-phase preparative plate and eluted with 0.05 to 0.1% TFA in water. More than 40 elutions were performed to try to achieve as much separation as possible. No distinct separation was observed with psilocybin essentially streaking up the plate. Nonetheless, three bands were removed from the preparative plate sampling from the top, middle and bottom of the psilocybin streak. These were all analyzed by UPLC and the band with the highest level of the targeted impurity is shown in Figure S7. Enrichment and ultimately isolation of the early eluting impurity at RRT 0.60 was not successful via use of a reversed-phase preparative plate. Each of the collected bands provided no separation with the impurity, psilocybin, and psilocin essentially co-eluting. Attention was then turned to an in-situ generation of the impurity.

**Figure S7:** UPLC trace of the bottom band from the reversed-phase preparative plate.

| Name | Retention Time | Area  | % Area | Height |
|------|----------------|-------|--------|--------|
| 1    | 1.623          | 497   | 0.60   | 178    |
| 2    | 1.823          | 5199  | 6.24   | 1663   |
| 3    | 1.978          | 2509  | 3.01   | 669    |
| 4    | 2.059          | 972   | 1.17   | 276    |
| 5    | 2.218          | 1135  | 1.36   | 203    |
| 6    | 2.488          | 612   | 0.73   | 96     |
| 7    | 2.663          | 86    | 0.10   | 31     |
| 8    | 2.847          | 358   | 0.43   | 172    |
| 9    | 2.903          | 20882 | 25.08  | 5561   |
| 10   | 3.264          | 1941  | 2.33   | 387    |
| 11   | 3.524          | 1961  | 2.35   | 446    |
| 12   | Impurity       | 3.699 | 12.06  | 1938   |
| 13   | 3.897          | 303   | 0.36   | 45     |
| 14   | 4.083          | 288   | 0.35   | 67     |
| 15   | 4.660          | 1635  | 1.96   | 182    |
| 16   | 4.797          | 575   | 0.69   | 79     |
| 17   | 4.883          | 340   | 0.41   | 53     |
| 18   | 5.065          | 344   | 0.41   | 30     |
| 19   | 5.668          | 606   | 0.73   | 56     |
| 20   | Psilocybin     | 6.001 | 31.83  | 2353   |
### In-situ generation of the impurity

In order to synthetically generate the unknown impurity at RRT 0.60, a 50 mg portion of the GMP batch (containing 0.10-0.11% impurity level) was treated with 2 mL of H₃PO₄ at room temperature to aid dissolution and then stirred for 30 minutes. Subsequently, POCl₃ (1 mL) was added at room temperature and the reaction mixture was stirred for a further 30 minutes. Reaction progression was followed by UPLC to monitor generation of the impurity. Reaction aliquots were quenched with 1.5 mL H₂O diluent for sample preparation.

Treatment of psilocybin with H₃PO₄ and POCl₃ was followed by UPLC analysis (Figure S8). A number of impurities were generated; however, the impurity of interest at RRT 0.60 reached a level of ~ 7.6% area with respect to the psilocybin area. Another early eluting peak was observed at ~ RRT 0.38 which could indicate potentially triple phosphorylation and the broad unresolved peak at ~ RT 11.5-12.5 minutes contained psilocin produced by *in situ* hydrolysis. The early eluting impurities were only generated under reasonably harsh reaction conditions and were not typically observed at such levels in the GMP manufacturing process and any low levels present were purged out during the work-up/purification phases. These results indicated that the impurity at RRT 0.60 could be synthesised *in-situ*; however, its isolation would require chromatography.

![Chromatogram of results generated from synthetic modification of psilocybin.](image)

### Enrichment of the impurity and acid catalyzed decay

In order to isolate the impurity at RRT 0.60, a small quantity (~ 20 mL) of the methanol filtrate purification stage of the GMP manufactured psilocybin was chromatographed using normal-phase silica. An eluent of 28-33% aqueous NH₃...
solution in MeOH at incremental gradients of 5%, 10%, 20%, 30%, 40% and 50% were used and 5 fractions were collected for each gradient mixture. The impurity of interest eluted between 30%-40% gradient and fractions in this range (showing > 90% impurity content) were mixed and concentrated under vacuum at 40 °C. After concentration, UPLC analysis was performed and showed a mixture of 60:40 between the impurity and psilocybin respectively (Figure S9). This change in ratio from the individual fractions was attributed to hydrolysis of the impurity to Psilocybin by the presence of H₂O catalysed in situ with generated H₃PO₄ and 40 °C bath temperature during the concentration process.

Figure S9: Chromatogram of enriched impurity at RRT 0.60.

In order to support the hydrolysis hypothesis, the above sample vial was further spiked with 150 Ml of conc12ntreated HCl and kept at 37 °C for 2 hours and then re-analyzed. The same vial was reinjected in 1.5 hour intervals up to 5 hours while held at 37 °C with UPLC data summarized in Table S8. The data showed a significant decrease in peak area for the RRT 0.60 impurity and a concomitant increase in psilocybin peak area with hydrolysis time. All other impurities showed insignificant change and were therefore omitted from the integration. It was surmised that the RRT 0.60 impurity, irrespective of known structure, was shown to hydrolyze to the pro-drug psilocybin in acidic aqueous conditions at physiological temperature.

| Time (hours) | Impurity at RRT 0.60 (% Area) | Psilocybin (% Area) | Psilocin (% Area) |
|--------------|-----------------------------|---------------------|-------------------|
| 0            | 58.82                       | 40.87               | 0.31              |
| 2            | 57.84                       | 41.83               | 0.33              |
| 3.5          | 55.84                       | 43.83               | 0.33              |
| 5            | 53.86                       | 45.81               | 0.33              |

Table S8: Conversion of RRT 0.60 impurity to psilocybin at 37 °C.
Enrichment of the impurity for structure elucidation

In order to isolate the impurity at RRT 0.60, a further portion of the methanol filtrate purification stage of the GMP manufactured psilocybin (~ 80 mL) was chromatographed using the previously described approach but using ammonia in methanol rather than aqueous ammonia to avoid unwanted hydrolysis. The obtained fractions were analyzed by UPLC and impurity enriched fractions were combined. The enriched fractions were concentrated under vacuum without applying heat to again negate potential hydrolysis. After concentration, UPLC analysis of the combined concentrated fractions was performed and is shown in Figure S10.

![Chromatogram of results generated from impurity enrichment for structure elucidation.](image)

**Figure S10:** Chromatogram of results generated from impurity enrichment for structure elucidation.

A successful enrichment of the impurity was achieved with > 92% overall purity. Only the peaks of interest are integrated, and all other impurities are omitted from the integration as they were not present in the currently manufactured GMP batch of psilocybin. This enriched sample was used to perform $^1$H NMR, $^{31}$P NMR and high-resolution MS analyses.

**NMR analysis of isolated/enriched impurity**

Both $^1$H and $^{31}$P NMR spectra were run on the enriched/isolated impurity with the data depicted in Figures S11 to S16 below. Although sample concentration was weak the data was quite conclusive in terms of structure elucidation. It should be noted that the $^1$H NMR contains residual methanol as the concentration process to isolate the impurity from silica chromatography was performed in the absence of heat to avoid hydrolysis of the impurity back to the parent pro-drug psilocybin. There are also some unrelated spurious peaks due to the signal to noise ratio and given the fact that the isolated purity is ca. 92% by UPLC.
Figure S11: $^1$H NMR in D$_2$O of isolated impurity RRT 0.60. D$_2$O residual solvent signal at 4.8 ppm and MeOH solvent signal from concentration of sample at 3.3 ppm.

Figure S12: $^1$H NMR in D$_2$O of isolated impurity RRT 0.60 and expanded aromatic region. A doublet, singlet, triplet and doublet pattern are apparent in the impurity similar to those on the indole skeleton of psilocybin.
Figure S13: \( ^1H \) NMR in D\(_2\)O of expanded alkyl region of the isolated impurity RRT 0.60. The ethylene CH\(_2\)CH\(_2\) chain is present at 3.2 ppm and 3.38 ppm but is overlapped by the residual methanol solvent signal at ca.3.3 ppm. The NMe\(_2\) signal is also present as a singlet at 2.87 ppm.

Figure S14: \( ^1H \) NMR in D\(_2\)O of isolated impurity RRT 0.60 (blue trace) overlay with psilocybin (red trace).
Figure S15: $^{31}$P NMR in D$_2$O of isolated impurity RRT 0.60. The $^{31}$P NMR spectra shows two coupling signals at -6 and -15 ppm with a coupling constant of 20 Hz consistent with P-O-P coupling. This can only be observed with phosphorylation on the psilocybin phosphate group. This coupling interaction is not possible elsewhere on the psilocybin skeleton.

Figure S16: $^{31}$P NMR in D$_2$O of isolated impurity RRT 0.60 (red trace) direct overlay with psilocybin (blue trace).
Conclusion

Both $^1$H and $^{31}$P NMR spectra indicate the impurity is structurally similar to psilocybin. The $^{31}$P NMR in particular is indicative of additional phosphorylation on the psilocybin phosphate group and this structural change also agrees with high resolution mass spectra. This indicates that the impurity (3-(2-(dimethylamino)ethyl)-1H-indol-4-yl trihydrogen diphosphate) is as shown in Figure S17 below. HRMS Calc. 365.0589; Found: 365.0677.

![Figure S17: Structure of RRT 0.60 impurity as indicated by NMR analysis.](image)

**Figure S17**: Structure of RRT 0.60 impurity as indicated by NMR analysis.