Whitepaper

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Microcrystal Electron Diffraction Study of Multi-component Salt and Cocrystal Forms of 3-Nitro-benzoic Acid and Pyrimethanil

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Abstract

Three single-phase crystals containing 3-nitrobenzoic acid (NBA) and pyrimethanil (PYR) in a 1:1 stoichiometry were found, and their structures were determined. This system (NBA•PYR), whose components have a ΔpK_a <1, can crystallize in one salt and two cocrystal forms. Structures were determined using both single-crystal X-ray diffraction (XRD) and microcrystal electron diffraction (microED). Both methods produced essentially equivalent crystallographic results that independently allow the solid form assignment of either salt or cocrystal. A remarkable result from the microED analysis was the complete crystal structure solution of a new polymorph of the cocrystal from a single impurity particle.

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Background

In 2015, Dr. Chris Frampton presented a talk at the Scientific Update 3rd Winter Process Chemistry Conference entitled "Salt-Cocrystal Interface Studies: The Importance of Being Hydrogen".[1] Studies of crystals formed between acids and bases whose ΔpK_a is less than 1 were described therein. That subject has received attention since the US Food and Drug Administration (FDA) clarified the different regulatory pathways necessary for approval of different salts and cocrystals of the same active pharmaceutical ingredients (APIs) when they are used in different drug products.[2] The FDA guidance establishes as a general guideline that the reaction of an acid–base pair having a $\Delta pK_a \geq 1$ will result in substantial proton transfer and produce a salt rather than a cocrystal. However, the guidance states that salt vs. cocrystal assignment can also be based on factual knowledge of the extent of proton transfer in the crystal structure determined using spectroscopic and other orthogonal approaches.

An interesting system described in the talk consists of 3-nitrobenzoic acid (NBA) and pyrimethanil (PYR) (Figure 1). The ΔpK_a of those compounds is 0.6, calculated by subtracting the pK_a of PYR (3.45) from that of NBA (4.05)[1]. It was reported that cooling solutions or suspensions of equimolar amounts of NBA and PYR, both white solids, in various organic solvents provided either yellow crystals, white crystals, or a mixture of the two. The yellow crystals were found to be a stoichiometric cocrystal while the white crystals were found to be a stoichiometric salt. The classification of cocrystal or salt was based on single-crystal X-ray diffraction (XRD) data, from which the location of the acidic NBA hydrogen atom was determined. In the cocrystal it is closest to the NBA oxygen atom while in the salt it is closest to the PYR nitrogen atom.

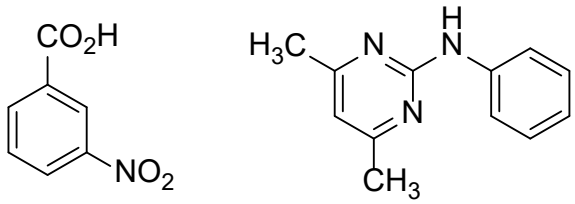


Figure 1. The structures of 3-nitrobenzoic acid (NBA, left) and pyrimethanil (PYR, right).

XRD provides highly accurate determinations of atomic coordinates. However, due to their relatively low atomic scattering factors, the exact positions of hydrogen atoms involved with hydrogen bonding, as in the case of cocrystals, or transfer, as in the case of salts, were

[1] Frampton, C. Salt–Cocrystal interface studies: The importance of being Hydrogen. Presented at Scientific Update 3rd Winter Process Chemistry Conference, University of Bath, Bath, UK, Dec. 14-16, 2015.
[2] Center for Drug Evaluation and Research, Regulatory Classification of Pharmaceutical Co-crystals. Guidance for Industry. United States Food and Drug Administration, Feb. 2018.

historically often inconclusive. Nowadays, using modern X-ray sources and detectors and given a good-quality crystal, the assignment can usually be made, as it was in the case of the NBA•PYR cocrystal and salt.

Microcrystal electron diffraction (MicroED) has become popular as an alternative technique to XRD for crystal structure determination. Electron diffraction can determine the crystal structure of much smaller crystals than required for XRD. The atomic scattering factor of hydrogen relative to carbon, nitrogen, and oxygen is relatively much larger. Therefore, at least in relative terms, microED should make it easier to determine the proton positions necessary to classify whether a crystalline phase is a cocrystal or a salt.

Although the phenomenon of electron diffraction by materials was discovered in 1927,[3] the technique was long overshadowed by XRD when it came to structure determinations.[4] X-rays, being uncharged and massless, interact with matter only weakly. They are scattered by the electron clouds surrounding atoms. Because of the weak interaction, interpretation of X-ray data can be done kinematically. That is, photons may validly be assumed to be scattered only once and do not cause disturbances in the material's electronic structure.

For electrons, which bear a negative charge, this assumption does not hold. They scatter because of strong coulombic interactions; electrostatic attraction toward protons in atomic nuclei and electrostatic repulsion from electron clouds surrounding the nuclei. Electrons interact with matter very strongly, leading to a greater contribution to dynamic scattering effects. Dynamical diffraction theory takes this into account, but the calculations involved are difficult and time-consuming. Recent developments using automatic data collection under continuous rotation reduce dynamical scattering.[4a] Therefore, this makes the use of kinematical refinement more valid, paving the way for the introduction of electron diffraction into common use. Recently, dedicated microED instruments have become commercially available.[5]

The fact that electrons interact strongly with materials, while a drawback to data interpretation, is advantageous in that only very small crystals are required to obtain measurable diffraction data. Crystals of sufficient size for X-ray analysis measure 25–150 μm in at least two dimensions, while microED analysis requires crystals only 50–500 nm in size, two to three orders of magnitude smaller. Multiple microcrystals from a single specimen can be analyzed quickly, about 5 minutes per crystal, and the data from crystals with identical structures can

[3] a) Davisson, C.; Germer, L. H. *Phys. Rev.* **1927**, *30*, 705−740, b) Davisson, C.; Germer, L. H. *Nature* **1927**, *119*, 558–560.
[4] Two reviews provide excellent historical accounts of structure determinations by electron *vs.* X-ray diffraction: a) Gemmi, M.; Mugnaioli, E.; Gorelik, T. E.; Kolb, U.; Palatinus, L.; Boullay, P.; Hovmöller, S.; Abrahams, J. P. *ACS Central Science* **2019**, *5*, 1315-1329, b) Saha, A.; Nia, S. S.; Rodríguez, J. A. *Chem. Rev.* **2022**, *122*, 13883–13914.
[5] Simoncic, P.; Romeijn, E.; Hovestreydt, E.; Steinfeld, G; Santiso-Quiñones, G.; Merkelbach, J. *Acta Cryst.* **2023**, *E79*, 410–422.

be merged to increase the completeness of the data set and the accuracy of the final results. Merging data sets also decreases the contribution of dynamical effects to a certain degree.

X-ray Diffraction Analyses

We repeated crystallization and XRD structure determination of both the NBA•PYR cocrystal and salt. Each single crystal was produced during crystallization of equimolar amounts of NBA and PYR by cooling solutions in 2-propanol. Each is a unique, single-phase, crystalline material containing a 1:1 ratio of NBA and PYR. Our data are consistent with those reported previously,[1] the yellow crystals are a cocrystal (CC1) and the white crystals are a salt (S1). Crystallographic data of the structures are shown in Table 1.

In each structure the asymmetric unit is the heterodimer shown in Figure 2. However, the conformations of the dimers differ slightly. In the cocrystal, all the aromatic rings are close to being coplanar, while in the salt the PYR phenyl ring is skewed out of the plane of the remaining aromatic rings. The in-plane coupling of the aromatic rings in the chromophore is likely the reason the cocrystal is yellow, while the salt is white. Packing diagrams are shown in Figure 3. NBA•PYR CC1 exhibits face-to-edge, or herringbone, packing, while NBA•PYR S1 consists of π–π stacked planes.

Table 1. Crystallographic Data from X-ray Diffraction Study.

Material	NBA•PYR CC1 (cocrystal)	NBA•PYR S1 (salt)
crystal appearance	yellow plate	colorless rod
crystal dimensions (mm)	0.21 × 0.20 × 0.06	0.23 × 0.07 × 0.06
radiation	Cu Kα (1.5418 Å)	Cu Kα (1.5418 Å)
temperature (K)	100	100
crystal system	monoclinic	triclinic
space group	$P\,2_1/n$	$P\,\overline{1}$
a (Å)	11.6846(5)	7.0853(10)
b (Å)	7.6167(4)	10.8038(11)
c (Å)	20.3464(7)	11.9447(13)
α (°)	90	106.174(8)
β (°)	104.361(3)	92.622(10)
γ (°)	90	99.247(11)
Z	4	2
volume (Å^3)	1,654.8(6)	862.78(18)
density (g/cm^3)	1.387	1.410
no. independent reflections	3395	3243
R factor (%)	4.38	8.18

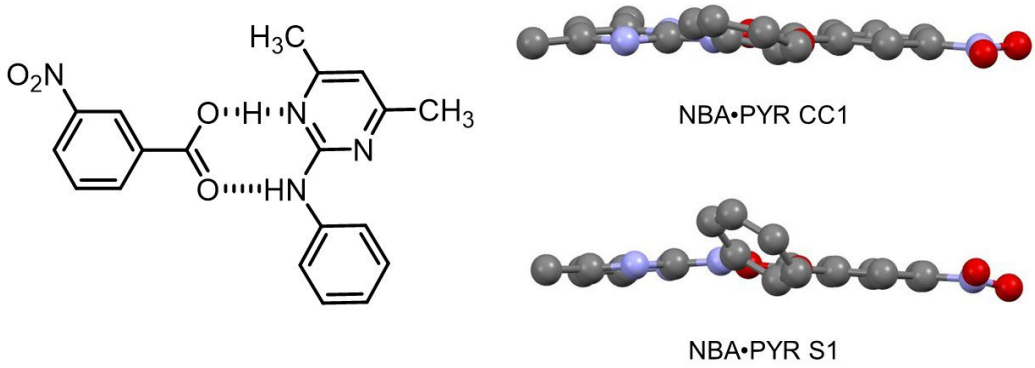
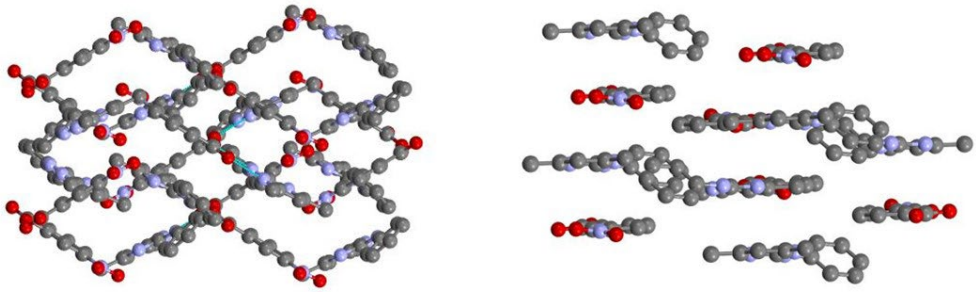


Figure 2. The asymmetric units of the cocrystal and salt.



NBA•PYR CC1 NBA•PYR S1

Figure 3. Packing diagrams of the cocrystal and salt.

As part of the structural analyses, care was taken to locate the hydrogen atoms from the NBA carboxyl group and the PYR amine group using difference Fourier analysis, and, in all cases, those atoms were freely refined without imposing restraints or idealizing their bonding. Using this approach, the position of the NBA acidic hydrogen atom should indicate if a structure is a salt or cocrystal based on whether it resides closer to the NBA oxygen atom or the PYR nitrogen atom. If the refinement were to fail, the result would be inconclusive. In this case, refinements were successful, and the positions of those protons were well resolved in the structures (Figure 4).

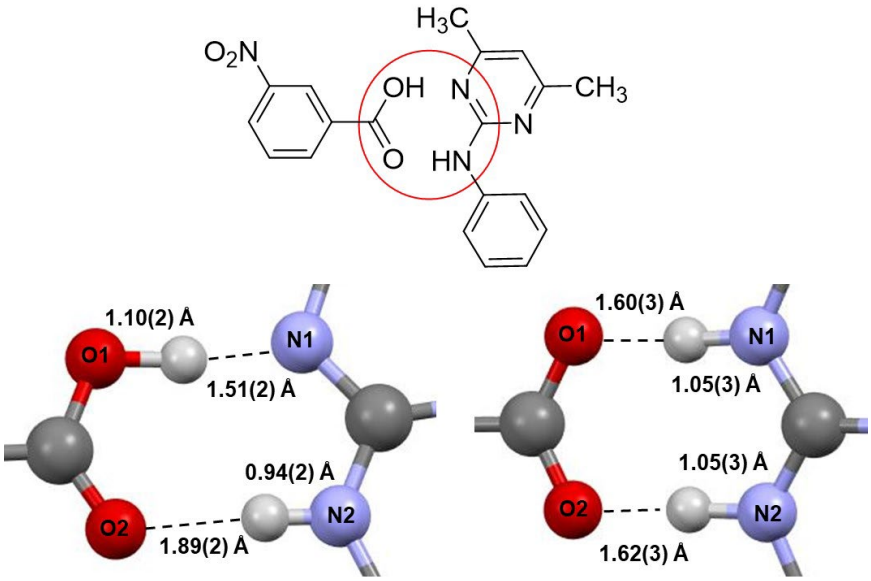


Figure 4. Interatomic distances from the X-ray crystal structures of NBA•PYR CC1 (left) and NBA•PYR S1 (right). Carbon atoms are dark gray, nitrogen atoms are blue, oxygen atoms are red, and hydrogen atoms are light gray. Hydrogen bonds are shown by black dashed lines.

In CC1, the proton remains closer to NBA than to PYR, indicating that it is a cocrystal. However, the proton is further from the NBA oxygen atom (1.10(3) Å) than the standard O–H bond distance reported for carboxylic acids, which is 1.015(17) Å based on neutron diffraction studies.[6] In S1, the proton is completely transferred to PYR, making it a salt. Note that both O–H hydrogen bonds and N–H covalent bonds are about the same length in that crystal. This illustrates the effect of the crystal structure on the ionizable proton positions. For compounds as close in pK_a as NBA and PYR are, proton positions used to make cocrystal or salt designations in crystalline solids are not controlled by ΔpK_a. Remember that pK_a values indicate the extent of ionization in an aqueous environment as a function of the acidity of the solution. The crystal structure offers a different environment for the molecules, causing them to exhibit different equilibrium behaviors for the acidic protons. The placement of the molecules in the crystal structures are identical in each of the three forms, offering the ability to form the same hydrogen bonds. However, the subtleties of the nearby molecular interactions and differences in molecular conformations apparently change the equilibrium behavior of the acidic proton in such a way that it prefers to transfer in one structure, making a salt. In contrast, it remains bonded to NBA in the other structure, making it a cocrystal.

[6] Allen, F. H.; Kennard, O.; Watson, D. G.; Brammer, L.; Orpen, A. G.; Taylor, R. *J. Chem. Soc., Perk II* **1987**, S1-S19.

Bulk samples of NBA•PYR CC1 and NBA•PYR S1 can be differentiated by powder X-ray diffraction (PXRD). Figure 5 shows overlay plots of PXRD patterns that were experimentally determined and calculated from the XRD data sets for NBA•PYR CC1 and NBA•PYR S1. Observed differences in the patterns are a result of the fact that the experimental patterns were measured at ambient temperature while the calculations from the single crystal XRD data utilized data collected at 100 K. Additionally, small peaks corresponding to CC2 are visible in the experimental pattern of CC1, indicating that a small amount of CC2 was in the sample analyzed.

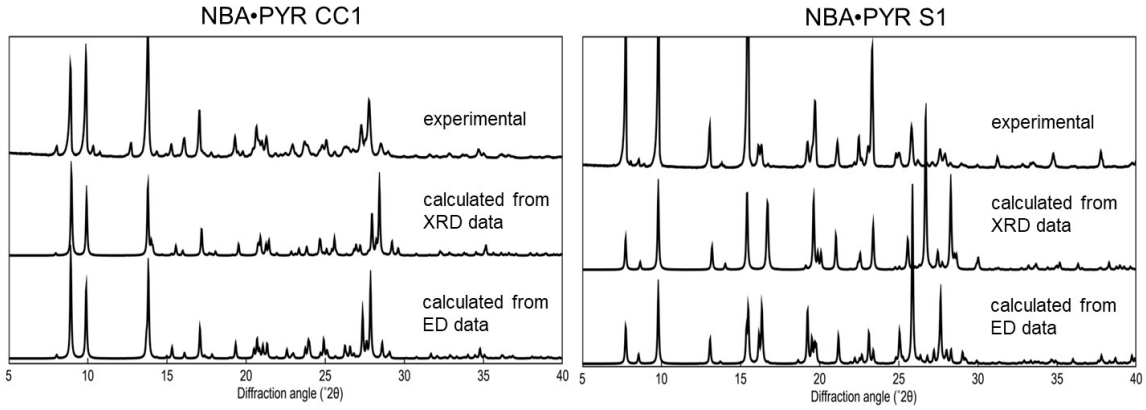


Figure 5. Overlay plots of experimentally determined and calculated PXRD patterns.

Electron Diffraction Analyses

Crystalline samples of NBA•PYR CC1 and NBA•PYR S1 were generated that were essentially phase-pure by XRPD analyses. A small amount of material from each sample was gently crushed between microscope slides, and the crushed material was brought into contact with a standard transmission electron microscopy grid (amorphous carbon on Cu). The specimens were analyzed at room temperature using an Eldico ED-1 electron diffractometer operating at an acceleration voltage of 160 kV (λ = 0.02851 Å). The grids were screened for crystals in STEM (scanning transmission electron microscopy) mode and diffraction data were recorded in continuous rotation mode with an electron beam of 750 nm diameter. For kinematical refinement, data were processed using the APEX4[7] software package and refined with ShelXL[8].

[7] APEX4, Version 2022.1-1; Bruker AXS Inc.: Madison, Wisconsin, USA, 2022.
[8] Sheldrick, GM. *Acta Cryst. A* **2008**, 64(1), 12–22.

Surprisingly, two structures were obtained from the yellow NBA•PYR CC1 specimen (Table 2). One is NBA•PYR CC1, whose structure is consistent with the structure solved by XRD. This was found in 10 out of 11 measured crystals.

One microcrystal in the specimen was found to have a different structure than NBA•PYR CC1. A full data set was obtained from that single crystal, and it was of sufficient quality for solution of the structure (Table 2). The new cocrystal, NBA•PYR CC2, has the same dimer as the asymmetric unit and packs in a herringbone fashion, very similar to NBA•PYR CC1 (see Figure 2 and Figure 3), but differs in that the aromatic rings are almost fully coplanar.

The structure obtained from the specimen of NBA•PYR S1 is the same as that determined by XRD (Table 2).

PXRD patterns calculated from the microED data are shown in Figure 5. The results are very similar as seen by comparing the calculated patterns to the experimental patterns. Note that the PXRD patterns calculated from the ED structures correspond slightly better to the experimentally determined PXRD patterns in peak positions than those calculated from XRD data. That is because both PXRD and microED measurements were performed at ambient temperature, whereas XRD data were collected at 100 K.

Table 2. Micro Electron Diffraction Data

sample	NBA•PYR CC1	NBA•PYR S1	NBA•PYR CC2
radiation	electrons (0.02851 Å)	electrons (0.02851 Å)	electrons (0.02851 Å)
temperature	ambient	ambient	ambient
crystal system	monoclinic	triclinic	monoclinic
space group	$P\,2_1/n$	$P\,\bar{1}$	$C\,2/c$
a (Å)	11.62(4)	7.26(4)	29.80(8)
b (Å)	7.87(2)	10.90(5)	7.34(2)
c (Å)	20.39(6)	11.92(6)	17.58(5)
α (°)	90	105.273(19)	90
β (°)	103.771(5)	94.64(3)	111.247(6)
γ (°)	90	99.22 (5)	90
Z, Z'	4, 1	2, 1	8, 1
volume (Å^3)	1811.05	890.669	3583.93
density (g/cm^3)	1.34	1.37	1.36
no. independent reflections	2105	2623	2227
R factor (%)	15.7	19.37	20.33

Hydrogen Placement

From X-ray diffraction data, hydrogen atoms are typically located after an initial refinement using Fourier difference maps during the crystal structure elucidation process. These maps show the location of electron density (in e/Å^3) unaccounted for by the current structural model. Placement of hydrogen atoms at positions of maximum electron density and further refinement provides a final structural model. This procedure works the same way with electron diffraction data, with the small difference that the map represents linear electron density, or electrostatic potential.[9]

Figure 7 shows the Fourier difference maps of the CC1 and S1 structures from ED data prior to placing the hydrogen atoms involved with the hydrogen bonding. The green meshes mark the regions within which the electron density is relatively high. The pink and blue markers with labels Q1 and Q2 indicate the location of the highest values within those regions, thus indicating the positions of the hydrogen atoms.

In the NBA•PYR CC1 structure, for the O1···H···N1 hydrogen, the electron density clearly indicates that the hydrogen atom is closer to O1. The maximum electron density value is at Q1. The O2···H···N2 hydrogen region is somewhat more dispersed, but the maximum peak, Q2, is closer to the N2 nitrogen atom than to O2. The location of the hydrogen positions clearly designates NBA•PYR CC1 as a cocrystal.

In the case of NBA•PYR S1, the residual electron density between the O1 and N1 atoms indicates that the hydrogen atom is closer to N1 (Q2). The location of that hydrogen position provides a clear designation of NBA•PYR S1 as a salt. The O2···H···N2 hydrogen atom (indicated by Q1) is closer to the N2 than to O2, although the electron density is dispersed somewhat more towards the center of the O2···N2 interaction.

After placing the hydrogen atoms, their positions could be freely refined and they stayed in their respective salt and cocrystal positions, confirming the cocrystal and salt assignments of the structures.

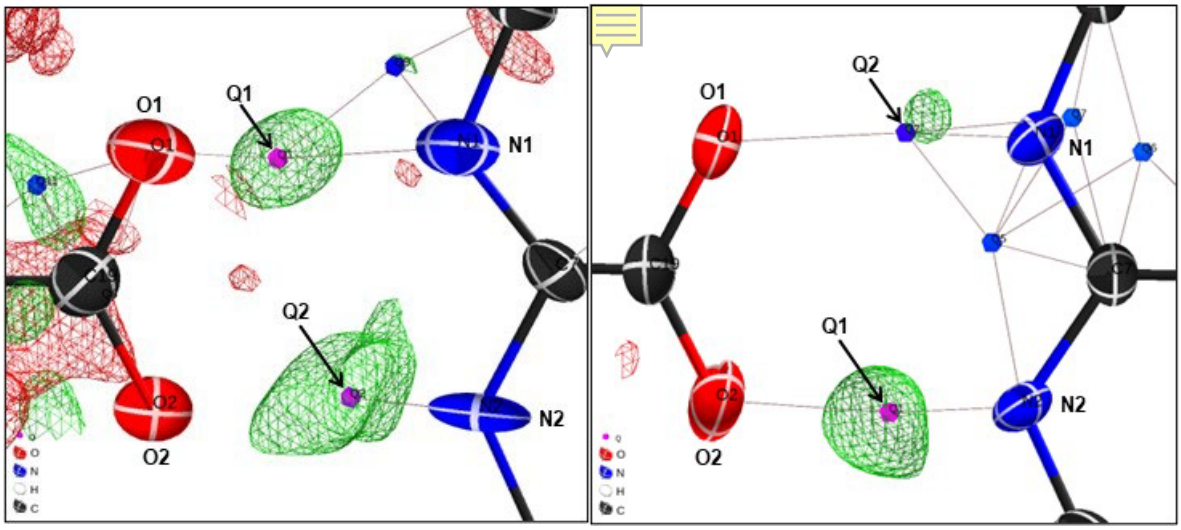


Figure 6. Difference Fourier maps for electron diffraction data used in placement of the hydrogen atoms in the NBA•PYR CC1 (left) and S1 (right) structures.

Comparison between X-Ray and Electron Diffraction Results

The estimated errors are listed in the tables in parentheses. A comparison of these errors shows that XRD results are generally more precise than ED results. Figure 8 shows a side-by-side image of the packing diagrams of the CC1 structures along the crystallographic *b* axis. This comparison shows that small, subtle differences may exist between the two methods, but the structures are identical overall. The same is true in comparison of packing diagrams for the S1 structures.

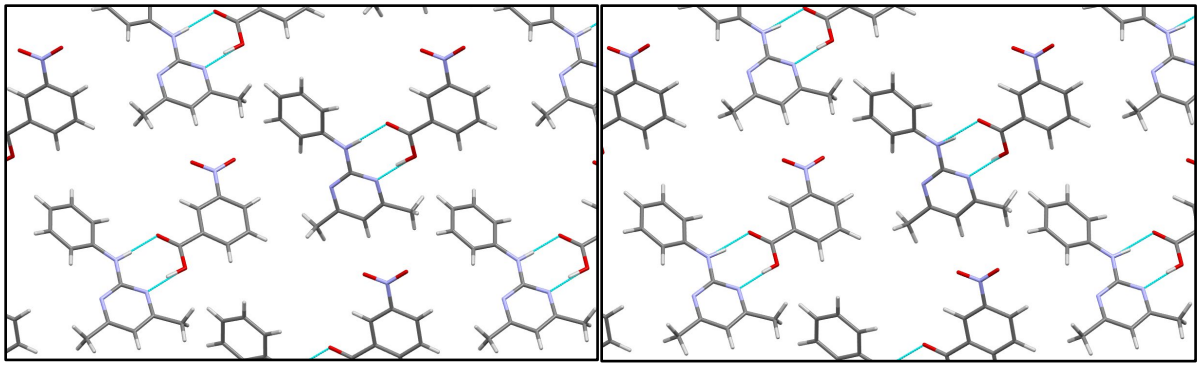


Figure 7. Comparison of Packing Diagrams for NBA•PYR CC1 from XRD (left) and ED (right)

Conclusion

The present study revisited the NBA•PYR system, known to be a cocrystal in one crystal structure and a salt in another. Both crystal structures were confirmed by microED, and the protons involved in the hydrogen bonding could be located in the microED data. In addition, from the same batch of material that contained mostly the previously elucidated polymorph CC1, a single microcrystal of a polymorphic cocrystal (CC2) was found, and its structure was solved.

Collected at room temperature and offering much less diffraction peak intensities than the XRD data, the microED data were still of sufficient quality to assign the solid forms as co-crystals and a salt. Dynamical refinement enhanced hydrogen atom positions, but this was not necessary to identify salt or cocrystal. That capability, coupled with the ease of sample preparation and speed of data collection, make microED an ideal technique for routine use in solving crystal structures. We have integrated the technique for early pharmaceutical development activities such as polymorph, salt, and cocrystal screening.

MicroED is also highly applicable in downstream processes, including detecting and identifying synthetic contaminants, metabolites, and infringing materials in legal and regulatory settings. Its ability to analyze extremely small quantities of material makes it invaluable for detecting trace amounts of crystalline impurities or unapproved compounds. Additionally, MicroED's utility extends to industries such as semiconductors and batteries, where the precise identification and characterization of phase impurities or novel materials can have significant impacts on product performance and safety. The technique’s rapid data collection and ease of sample preparation make it an ideal tool for these applications, ensuring reliable, high-precision results even at the nanoscale.

Contributors

The original report describing preparation and XRD structure determination of NBA•PYR CC1 and S1 was made by Dr. Chris Frampton, Rbar3, Cambridge, UK.[1]

For this white paper, data were obtained from crystals prepared at Triclinic Labs, Lafayette, IN. Single crystal XRD structure determinations were carried out by Dr. Matthias Zeller, Purdue University, West Lafayette, IN, USA. MicroED structure determinations were carried out by Dr. Christian Jandl, ELDICO Scientific, Allschwil, Switzerland.

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