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Complex Transitions between Dihydrate and Anhydrate Forms of Ectoine - Unexpected Behavior of A Highly Hygroscopic Compatible Solute in the Solid State

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1. Experimental Procedures

1.1. Crystallization of ectoine

The single-crystals for the X-ray and neutron diffraction experiments were grown by isothermal evaporation of the solvent from a solution of ectoine. Anhydrous ectoine was purchased from bitop and used as obtained. All solvents were purchased in *p.a.* quality and also used without further purification. For the crystallization of ectoine dihydrate, 100 mg (0.703 mmol) ectoine were dissolved in 100 mL water by heating. After cooling down to ambient temperature, the solvent was allowed to slowly evaporate yielding small crystals that were used as seeds for a second crystallization. For the second crystallization, a stock solution of 3,200 mg (22.5 mmol) ectoine in 3520 µL water was prepared. 50 µL of this solution and a seeding crystal were added to each well of a 96-well plate. By slow evaporation of the solvent at ambient temperature colorless crystals of ectoine dihydrate were obtained. For the crystallization of ectoine anhydrate, 11.0 mg (0.077 mmol) ectoine were dissolved in 300 µL *N,N*-dimethylformamide (DMF). The solvent was allowed to slowly evaporate at ambient temperature (293 – 296 K) yielding colorless single-crystals of ectoine anhydrate within ten days.

1.2 Single-crystal X-ray diffraction - Data collection and refinement

Single crystal data were collected on a Bruker Smart three-circle diffractometer equipped with an Incoatec IµS Cu microfocus source with mirror optics and an APEX CCD detector. Data collection and reduction was performed with APEX3[1]. The crystal structure was solved by direct methods using SHELXS[2] and refined with SHELXL[3]. All H atoms were initially located by difference Fourier synthesis. Subsequently, all H atoms bound to C atoms were refined using a riding model with the methyl C-H distances constrained to 0.98 Å and the aromatic C-H distances constrained to 0.95 Å. Their isotropic displacement parameters were set to $U_{iso}(H) = 1.5\ U_{eq}(C)$ for methyl H atoms and $1.2\ U_{eq}(C)$ for aromatic H atoms. For the H atoms of the methyl groups, free rotation about their local threefold axis was allowed. The coordinates of H atoms bonded to N atoms were refined with the N-H distances restrained to 0.88 (2) Å. Their isotropic displacement parameters were coupled to the equivalent isotropic displacement parameters of the parent N atoms, with $U_{iso}(H) = 1.2\ U_{eq}(N)$. The coordinates of the H atoms of the water molecules were refined as well with the O-H distances restrained to 0.84 (1) Å and the H···H distances restrained to 1.4 (1) Å. Their isotropic displacement parameters were coupled to the equivalent isotropic displacement parameters of the O atoms, with $U_{iso}(H) = 1.2\ U_{eq}(O)$. All non-H atoms were refined anisotropically.

1.3. Single-crystal neutron diffraction - Data collection and refinement

Single crystal data were collected at the thermal neutron diffractometer for single-crystal and fibre diffraction, D19, at Institute Laue-Langevin, Grenoble, France. Neutrons with a wavelength of 1.1698 Å were used. The reflection intensities were collected by omega scans mode with typical scan-width of 80° and 0.07°/step. The ILL program Multi Detector Acquisition Software was used for data collection. The determination of the unit cell and the processing of the raw data was performed with DIRAX (determination of the unit cell), RETREAT[4] and RAFD19 (data processing and refinement of the unit cell parameters). An absorption correction was applied with the ILL software D19abs. The structure was solved by using the phases obtained from the crystal structure determined by X-ray diffraction (see 1.2.). The structure was refined with SHELXL.

1.4. X-ray powder diffraction - Data collection

X-ray powder diffraction measurements were performed on a STOE Stadi-P diffractometer equipped with a Ge(111) monochromator and a linear position-sensitive detector (PSD) using Cu $K_{\alpha 1}$ radiation (λ = 1.54056 Å) and for the low temperature XRPD measurements equipped with an Oxford Cryostream 800. The samples were measured in borosilicate glass capillaries with a diameter of 0.7 mm or 1.0 mm. The data were collected using WinXPOW software 3.4[5].

Specific parameters for samples:
- decomposed sample
    - 0.7 mm capillary
    - Data collection at room remperature
    - Range: 2-80°/2Th
    - 150s/step, psd-step 0.2 = 5250s/step
    - Overall time for data collection: approx. 18.5 hours

- ectoine bitop
    - 0.7 mm capillary
    - Data collection at 173 K
    - Range: 3-60°/2Th
    - 60s/step, psd-step 0.5 = 720s/step
    - Overall time for data collection: approx. 2 hours

- after moisture uptake
- after 7 days
- after 10 days
    - 1.0 mm capillary
    - Data collection at 173 K

- Range: 3-80°/2Th
- 150s/step, psd-step 0.2 = 4650s/step
- Overall time for data collection: approx. 18 hours

- before DTA / TG
- after DTA / TG 60 °C
- after DTA / TG 115 °C
  - 0.7 mm capillary
  - Data collection at 173 K
  - Range: 3-80°/2Th
  - 150s/step, psd-step 0.2 = 4650s/step
  - Overall time for data collection: approx. 18 hours

- after DTA / TG 300 °C
  - 0.7 mm capillary
  - Data collection at room remperature
  - Range: 2-60°/2Th
  - 60s/step, psd-step 0.5 = 840s/step
  - Overall time for data collection: approx. 2.5 hours

1.5. DTA/TG study on ectoine dihydrate

Differential thermal and thermogravimetric analyses (DTA/TG) were performed on a SETARAM (TGA 92) device. The samples of about 5 mg were filled into Al_2O_3 crucibles and measured under argon atmosphere with a heating rate of 10 K min^{-1} and a constant flow rate of about 75 mL min^{-1}. The heating ranges were from 293 K to 573 K, to 333 K or to 388 K, respectively.

Samples:
- Heating to 573 K
  - Heating rate: 10 K/min
  - Ar-Atmosphere
  - Initial mass of sample: 3.313 mg

- Heating to 333 K
  - Heating rate: 10 K/min
  - Ar-Atmosphere
  - Initial mass of sample: 4.944 mg

- Heating to 388 K
  - Heating rate: 10 K/min
  - Ar-Atmosphere
  - Initial mass of sample: 6.676 mg

1.6. Study of the water uptake of anhydrous ectoine via the gas phase

For the study of the uptake of moisture by ectoine from the air, 50 mg of anhydrous ectoine were placed in a 1 mL vial. This vial was left open and was placed inside a 10 mL vial containing 3 mL of water. The outer vial was capped with a polyethylene cap and sealed with Parafilm. After 24 hours, the small inside vial was removed from the larger one, capped with a polyethylene cap and sealed with Parafilm and stored for 2 days (over weekend) at ambient conditions. For the XRPD measurement, a part of the sample was filled in a capillary (diameter 0.7 mm) which was sealed by melting it off. After the XRPD measurement the sealed capillary of this sample was stored at constant temperature of approximately 27 °C. After seven days and ten days, respectively, powder patterns were collected again in order to determine phase changes.

1.7. Computational study

All geometries throughout this paper were treated on the level of Møller-Plesset perturbation theory (RI-MP2) with the TZVPP basis set. [6,7] The Resolution of Identity (RI) approximation[8–10] was applied to the MP2 correlation integrals. The conductor-like screening model (COSMO)[11] with an epsilon of 10 was used. All calculations were done with ORCA 3.0.3.[12] To correct for the basis set superposition error (BSSE) in the adiabatic interaction energies of the dimers, the site-site-function-counterpoise correction was applied.[13,14] As input structures, we cut dimer structures from the experimentally determined crystal structures. In order to compute the corresponding energies, single point calculation without further geometry optimization were performed. For the dihydrate without water, meaning the ectoine with the carboxylate group in an axial position, simply the water molecules were deleted form the corresponding crystal structure.

2. Results and Discussion

2.1 Crystal Structure of Ectoine Dihydrate

Ectoine dihydrate (ECT · 2H$_2$O; CCDC 1890936) crystallized in the orthorhombic space group $P2_12_12_1$ with one ectoine zwitterion and two water molecules in the asymmetric unit (Figure S1). The carboxylate group of the ectoine zwitterions adopts an axial conformation (Table S1) and they form a three-dimensional hydrogen bonded network *via* N—H···O hydrogen bonds [Figure S2 (a) and Table S2]. The water molecules are linked to the ectoine zwitterions by O—H···O hydrogen bonds and are located in channels running along the c-axis [Figure S2 (b) and (c)].

Figure S1. A perspective view of ECT · 2H$_2$O, showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level. Dashed lines indicate hydrogen bonds.
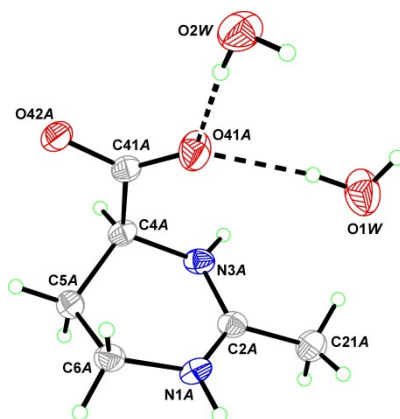

Figure S2. A partial packing diagram for ECT · 2H$_2$O, showing the connection of the ectoine zwitterions *via* N—H···O hydrogen bonds [part (a)], the connection between the water molecules and the ectoine zwitterions *via* O—H···O hydrogen bonds [part (b)], and the resulting channel-like arrangement along the *c*-axis of the ectoine zwitterions with the water molecules located in the channels [part (c)]. Dashed lines indicate hydrogen bonds. Symmetry codes: (i) −x+3/2, −y+1, z+1/2; (ii) x−1/2, −y+3/2, −z+1; (iii) x−1/2, −y+1/2, −z+1.
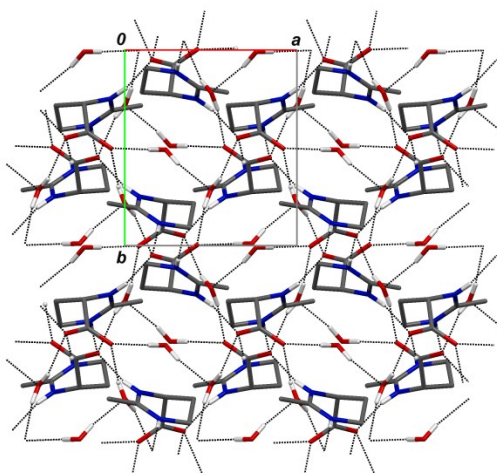
(a)
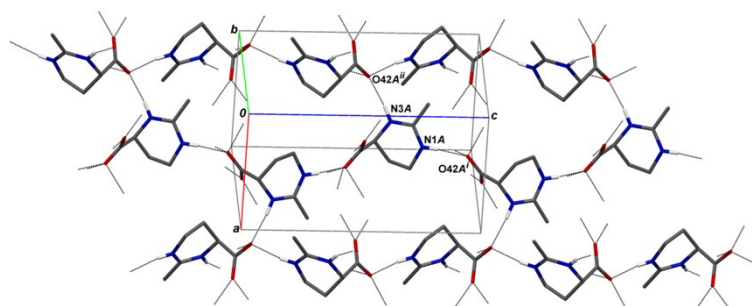
(b)
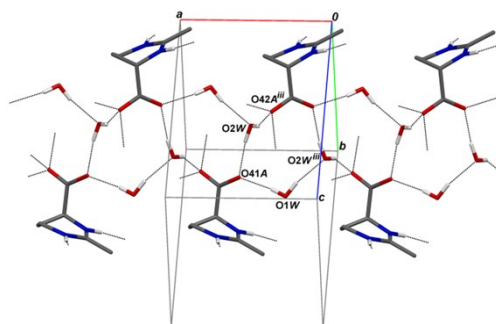
(c)

Table S1. Selected torsion angle (º) for (ECT · 2H$_2$O)

Atoms	Tors
C2A - N3A - C4A - C41A	90.1 (2)

Table S2. Hydrogen-bond geometry (Å, º) for (ECT · 2H$_2$O)

D—H···A	D—H	H···A	D···A	D—H···A
N1A—H1A···O42A[i]	0.85 (2)	1.98 (2)	2.816 (2)	170 (2)
N3A—H3A···O42A[ii]	0.88 (2)	1.94 (2)	2.809 (2)	167 (2)
O1W—H11W···O41A	0.87 (1)	1.96 (1)	2.823 (2)	172 (3)
O1W—H12W···O2W[iii]	0.86 (1)	1.96 (1)	2.804 (3)	168 (3)
O2W—H21W···O41A	0.85 (1)	1.92 (1)	2.761 (2)	171 (3)
O2W—H22W···O42A[iii]	0.85 (1)	2.21 (1)	3.022 (2)	158 (2)

Symmetry codes: (i) −x+3/2, −y+1, z+1/2; (ii) x−1/2, −y+3/2, −z+1; (iii) x−1/2, −y+1/2, −z+1.

2.2. Crystal Structure of Ectoine Anhydrate

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Ectoine anhydrate (ECT, CCDC 1890935) crystallized in the monoclinic space group $P2_1$ with two ectoine zwitterions in the asymmetric unit (Figure S3). The carboxylate groups of the ectoine zwitterions adopt an equatorial conformation (Table S3) yielding a two-dimensional hydrogen bonded network stabilized by N—H⋯O hydrogen bonds [Figure S4 and Table S4].

Figure S3. A perspective view of ECT, showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level. Dashed lines indicate hydrogen bonds.
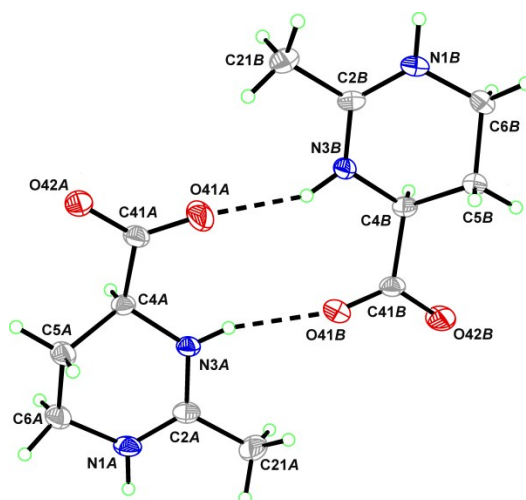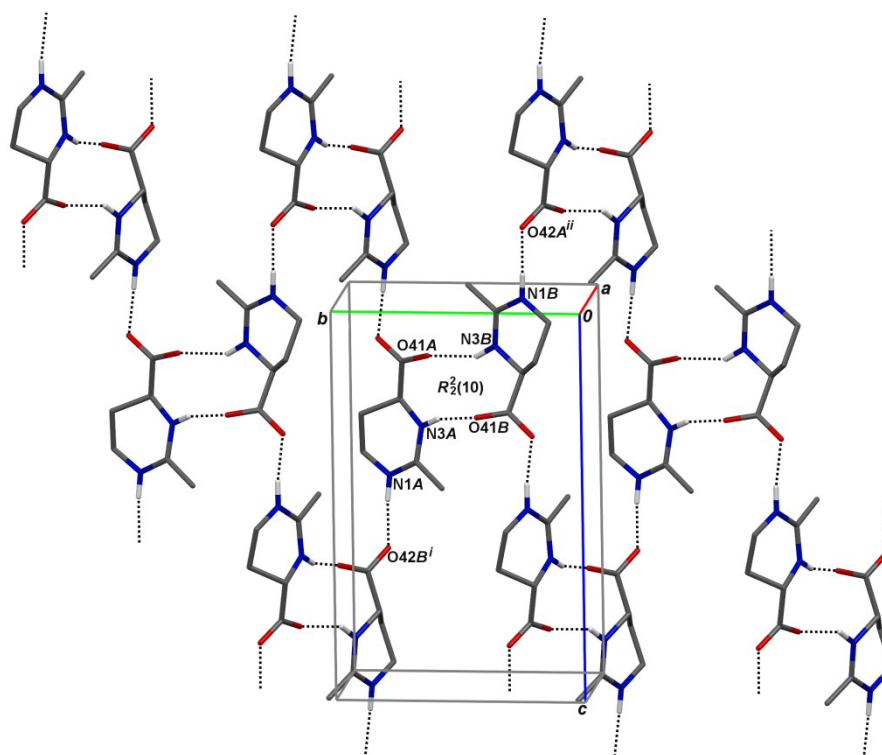

Figure S4. A partial packing diagram for ECT, showing the connection of the ectoine zwitterions *via* N—H⋯O $R^2_2(10)$ hydrogen bonds [part (a)], and the resulting undulated layer-like arrangement of the ectoine zwitterions [part (b)]. Dashed lines indicate hydrogen bonds. Symmetry codes: (i) −x+1, y+1/2, −z+1; (ii) −x+2, y−1/2, −z.
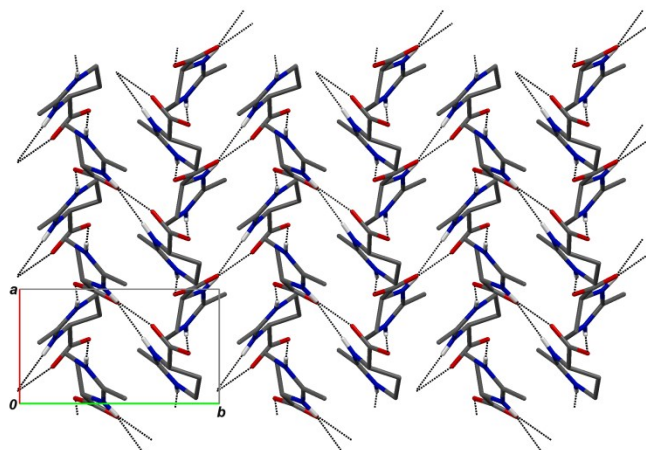
(a)

(b)

Table S3. Selected torsion angles (º) for (ECT)

Atoms	Tors
C2A - N3A - C4A - C41A	160.8 (3)
C2B - N3B - C4B - C41B	158.6 (3)

Table S4. Hydrogen-bond geometry (Å, º) for (ECT)

D—H···A	D—H	H···A	D···A	D—H···A
N1A—H1A···O42B[i]	0.90 (2)	1.83 (2)	2.715 (3)	171 (4)
N3A—H3A···O41B	0.85 (2)	2.08 (2)	2.908 (3)	164 (3)
N1B—H1B···O42A[ii]	0.88 (2)	1.83 (2)	2.703 (3)	178 (4)
N3B—H3B···O41A	0.87 (2)	2.05 (3)	2.857 (4)	153 (3)

Symmetry codes: (i) −x+1, y+1/2, −z+1; (ii) −x+2, y−1/2, −z.

Table S5. Experimental details of the single-crystal X-ray diffraction analysis.[19]

Crystal data

Chemical formula	$C_6H_{10}N_2O_2 \cdot 2(H_2O)$	$C_6H_{10}N_2O_2$
M_r	178.19	142.16
Crystal system, space group	Orthorhombic, $P2_12_12_1$	Monoclinic, $P2_1$
Temperature (K)	173	173
a, b, c (Å)	7.5940 (3),	5.21733 (4),
	8.6265 (5),	9.11328 (7),
	13.3619 (6)	14.35413 (11)
α, β, γ (°)	90,	90,
	90,	90.7911 (3),
	90	90
V (Å³)	875.33 (7)	682.43 (1)
Z, Z'	4, 1	4, 2
Radiation type	Cu $K\alpha$	Cu $K\alpha$
μ (mm⁻¹)	0.96	0.88
Crystal size (mm)	0.36 × 0.22 × 0.16	0.33 × 0.26 × 0.15
Data collection		
Diffractometer	Siemens CCD three-circle diffractometer	Siemens CCD three-circle diffractometer
Absorption correction	Multi-scan *SADABS*[15] (Sheldrick, 1996)	Multi-scan *SADABS*[15] (Sheldrick, 1996)
T_{min}, T_{max}	0.914, 1.000	0.897, 1.000
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	12908, 1575, 1575	10356, 2230, 2229
R_{int}	0.026	0.023
$(\sin \theta/\lambda)_{max}$ (Å⁻¹)	0.608	0.609
Refinement		
$R[F^2 > 2\sigma(F^2)]$, wR(F²), S	0.028, 0.075, 1.10	0.041, 0.112, 1.17
No. of reflections	1575	2230
No. of parameters	128	195
No. of restraints	8	5
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}, \Delta\rho_{min}$ (e Å⁻³)	0.14, −0.13	0.27, −0.36
Absolute structure	Flack x determined using 623 quotients [(I+)-(I-)]/[(I+)+(I-)]^16 (Parsons, Flack and Wagner, Acta Cryst. B69 (2013) 249-259).	Flack x determined using 907 quotients [(I+)-(I-)]/[(I+)+(I-)]^16 (Parsons, Flack and Wagner, Acta Cryst. B69 (2013) 249-259).
Absolute structure parameter	−0.04 (4)	0.06 (9)

Computer programs: *APEX3*[1], *SHELXS*[3][2], *SHELXL*[3], *Mercury*[17] and *XP* in *SHELXTL-Plu*[2], *publCIF*[18].

Table S6. Experimental details of the single-crystal neutron diffraction analysis.[19]

Crystal data	
Chemical formula	$C_6H_{14}N_2O_4$
M_r	178.19
Crystal system, space group	Orthorhombic, $P2_12_12_1$
Temperature (K)	173
a, b, c (Å)	7.5940 (3), 8.6265 (5), 13.3619 (6)
V (Å^3)	875.33 (7)
Z	4
Radiation type	Neutrons, λ = 1.1698 Å
μ (mm^{-1})	0.04
Crystal size (mm)	1.00 × 1.00 × 1.00

Data collection	
Diffractometer	4 circle diffractometer D19, ILL, Grenoble, France
Absorption correction	Gaussian ILL program D19abs
T_{min}, T_{max}	0.434, 0.730
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	7579, 2971, 2909
R_{int}	0.063
$(\sin\theta/\lambda)_{max}$ (Å^{-1})	0.759

Refinement	
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.032, 0.081, 1.15
No. of reflections	2971
No. of parameters	292
No. of restraints	220
H-atom treatment	All H-atom parameters refined
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.57, −0.75
Absolute structure	All f" are zero, so absolute structure could not be determined

Computer programs: ILL program Multi Detector Acquisition software, ILL program *RAFD19*, ILL program *RETREAT*, *SHELXL*[3].

Table S7. Hydrogen-bond geometry (Å, º) for ectoine-dihydrate from single-crystal neutron diffraction analysis (CCDC 1955570).[19]

D—H$\cdots A$	D—H	H$\cdots A$	$D\cdots A$	D—H$\cdots A$
N1A—H1$A\cdots$O42A^i	1.029 (3)	1.802 (3)	2.8185 (16)	168.5 (3)

N3*A*—H3*A*···O42*A*[ii]	1.026 (3)	1.805 (3)	2.8095 (17)	165.1 (3)
O1*W*—H11*W*···O41*A*	0.954 (4)	1.870 (4)	2.822 (3)	176.7 (4)
O1*W*—H12*W*···O2*W*[iii]	0.963 (4)	1.864 (4)	2.812 (3)	167.2 (4)
O2*W*—H21*W*···O41*A*	0.971 (4)	1.791 (4)	2.762 (2)	177.6 (4)
O2*W*—H22*W*···O42*A*[iii]	0.961 (4)	2.093 (4)	3.003 (2)	157.3 (4)

Symmetry codes: (i) −*x*+3/2, −*y*+1, *z*+1/2; (ii) *x*−1/2, −*y*+3/2, −*z*+1; (iii) *x*−1/2, −*y*+1/2, −*z*+1.

2.3. X-ray powder diffraction study

Figure S5. Powder pattern of the powder remaining from a single-crystal of ECT $\cdot$ 2H$_2$O after exposure to air over night.
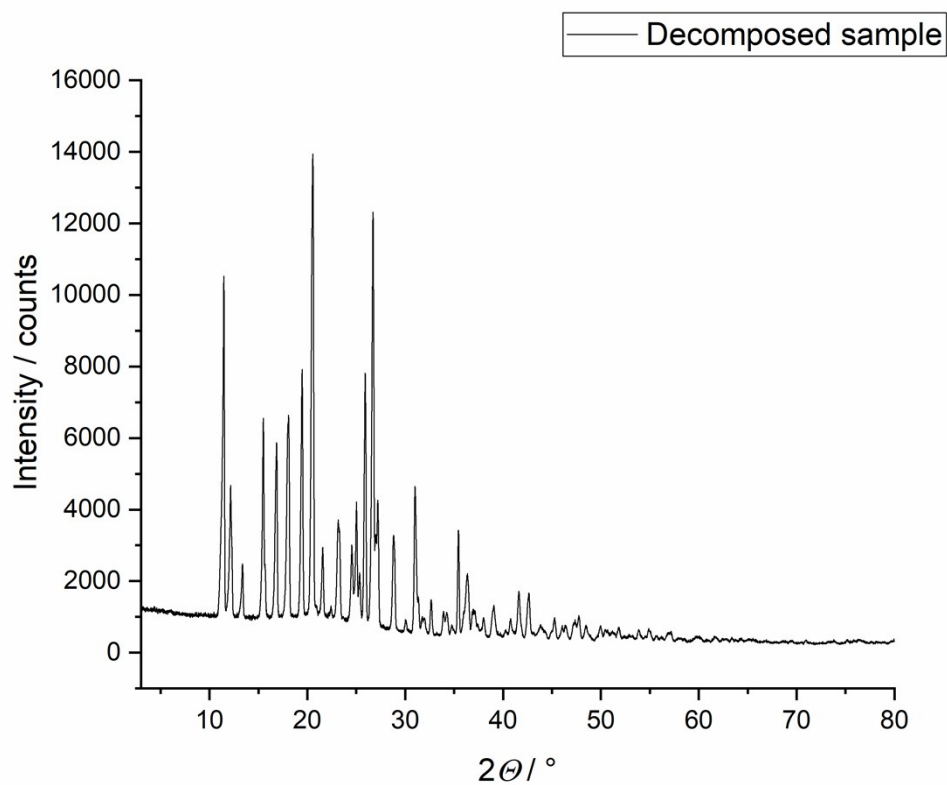

Figure S6. Comparison of the simulated powder pattern of ECT[19] and a sample of ectoine from bitop..
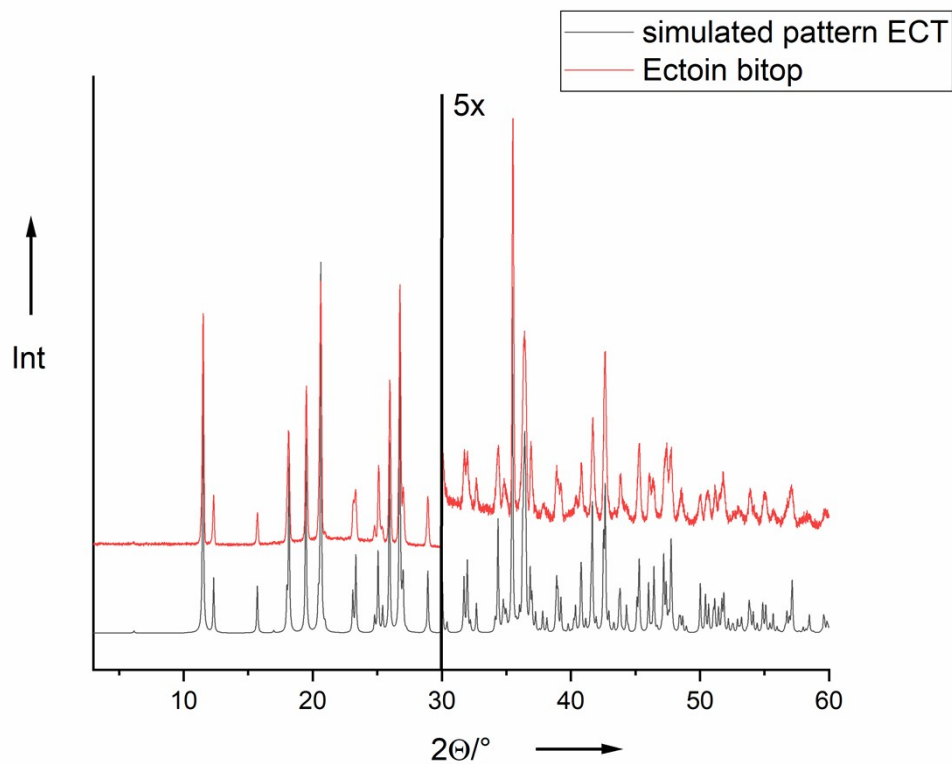
Figure S7. Comparison of the simulated powder patterns (from single crystal structure)[19] of ectoine dihydrate (1) and ectoine anhydrate (2), with the experimental powder patterns of anhydrous ectoine from bitop, a sample of ectoine dihydrate before TGA (4), after heating in DTA / TG to 60 °C (5), after heating in DTA/ TG to 115 °C (6), and after heating in TGA to 300 °C (7). At 60 °C, the sample is still the dihydrate, at 115 °C it comprises of a mixture of dihydrate and anhydrate and at 300 °C the sample is fully converted to the anhydrate.
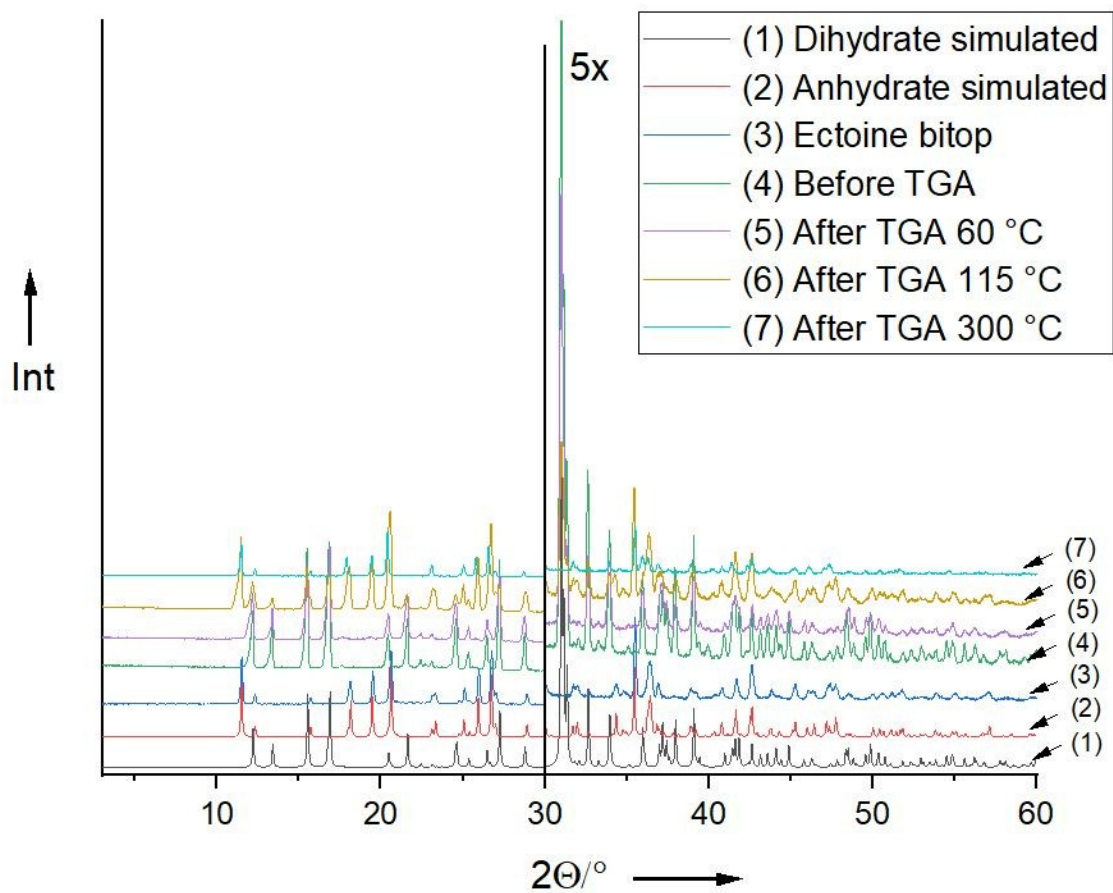
2.4. DTA / TG study on ectoine dihydrate

Figure S8. Results of the TG analysis on ectoine dihydrate.

M (Ectoine):	142.22 mg/mmol
M (Water):	18.02 mg/mmol
M (Ectoine dihydrate):	178.26 mg/mmol
Initial mass of sample:	3.313 mg or 0.0186 mmol Ectoine dihydrate
TG mass at maximum:	0.369 mg
TG mass at minimum:	-0.327 mg
Overall ΔTG:	0.696 mg or 0.0386 mmol water
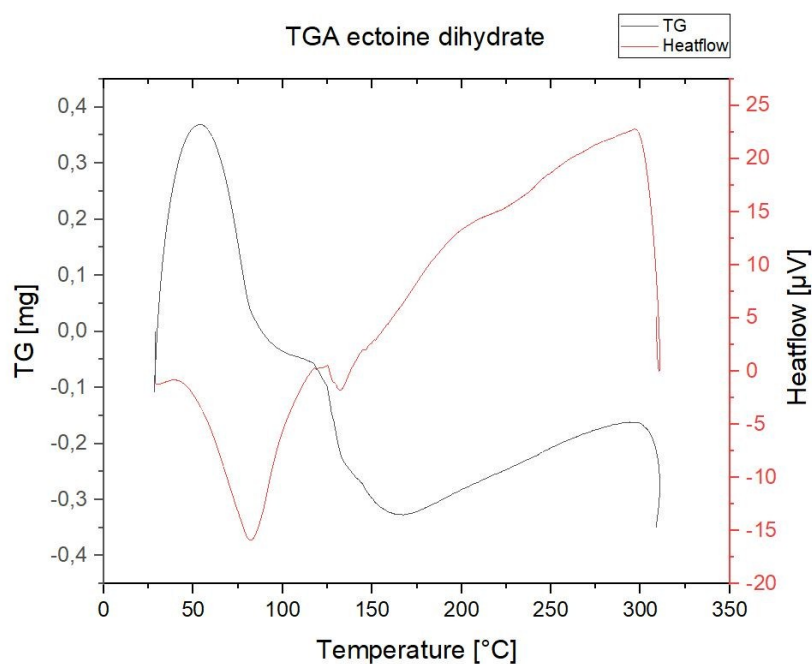
Note: The apparent increase of the sample's weight between 293 K and 323 K is related to the device. With increasing temperature, the density of the inert gas in the heating chamber decreases reducing the buoyancy of the sample holder on the balance thus causing an apparent increase of the sample's weight. The weight loss is usually calculated starting at the inflection point of the TG curve, in this case at approximately 323 K.

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19 CCDC 1890935, 1890936 and 1955570 contain the supplementary crystallographic data for this paper. These data are provided free of charge by The Cambridge Crystallographic Data Centre.

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