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Cocrystals and a Salt of the Bioactive Flavonoid: Naringenin

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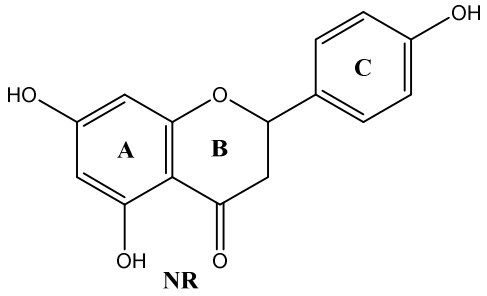
ABSTRACT:

Cocrystallization studies on Naringenin with 27 coformers have led to the formation of five new solid forms: piperazinium salt (PIP^+-NR^-) and four cocrystals with the coformers flavone (FLV), 4-hydroxypyridine (4-HP), anthranilamide (ATA), and 4,4′-bipyridine (Bipy). Structural characterization reveals that the hydrogen bonded head to tail dimer motif in naringenin is maintained only in the cocrystal with flavone (NR-FLV). All four neutral cocrystals maintain the S(6) O–H···O=C intramolecular hydrogen bond seen in naringenin with this carbonyl oxygen atom bifurcated. The piperazinium salt is the only structure in which the ether oxygen is involved in hydrogen bonding. Some of the structures display disorder in the chiral center of naringenin, making them anomalous racemic solid solutions. The solubility study revealed that the salt formation has significantly enhanced the solubility of naringenin, for example the piperazinium salt has enhanced solubility of >3,000 times that of the neutral parent compound.

Introduction:

Flavonoids are of considerable interest due to their abundant availability in various dietary substances like fruits, vegetables, nuts, and beverages such as coffee, tea, and red wine, as

well as in medicinal herbs.[1] It has been proven that they can act as effective scavengers of radicals *in vitro*,[2-3] and they have potential health benefits such as cardioprotection,[4-6] cerebrovascular and neuroprotection[7-9] as well as chemoprevention.[10] It is estimated that the daily oral dietary intake of the flavonoids is about 0.5-1.0 g in Western countries.[11] However, due to their poor solubility and bioavailability, it is difficult to formulate them in any potential medicine. One way to ameliorate the solubility of poorly-soluble APIs is the use of multi-component forms such as salts and cocrystals. Co-crystallization is evolving as an excellent paradigm to modulate/improve the physicochemical properties substantially by using non-covalent interactions. Cocrystallization can alter various properties such as melting point, stability, solubility, dissolution, friability, density and bioavailability of active pharmaceutical ingredient in a controlled way.[12-17] Converting a compound into a salt is a well-known conventional method in the pharmaceutical sector to improve various properties and it is estimated that over half of the medicines on the market are administered as salts.[18-20] Converting flavones into their corresponding salts is challenging due to the weakly acidic phenolic functional groups.



NR

Figure 1. Molecular structure of naringenin (NR). A, B and C corresponds to the commonly used notation for numbering the rings in naringenin[22]

Naringenin (NR) is a flavonoid widely available in citrus and tomato peel, Figure 1.[21] It exhibits a variety of biological activities including anti-inflammatory,[23] anti-oxidant,[24] anti-potent,[25-26] anti-allergetic,[27] phytoestrogenic,[28] anti-atherogenic,[29] and anti-ulcer activity.[30-33] Naringin is a glycoside of naringenin that readily hydrolyses in the intestinal epithelium to give naringenin.[34] However naringenin is reported to have very low solubility[35] and bioavailability[36] issues and, therefore, it was selected for this study.

To date, only three crystal structures associated with naringenin have been published in the literature. The first two are the structures of (±)-naringenin (CSD REFCODE is DOLRIF) [37]

and of the (±) solid solution (DOLRIF01).[38] The last structure is a salt with diazobicyclooctane (COLHER).[39]

In this study, a total of 27 different coformers selected from a selection of nutraceuticals, drugs, generally recognized as safe (GRAS) and non-GRAS molecules were investigated for their ability to form a cocrystal or salt with naringenin, as shown in Figure 2 [carboxylic acids (top), *N*-substituted heterocycles (middle) and neutral compounds (bottom)].

Five novel forms were discovered. Four of the forms are cocrystals; namely naringenin with flavone (NR-FLV, **18**), 4-hydroxypyridine (NR-4HP, **11**), anthranilamide (NR-ATA, **27**) and 4,4′-dipyridyl (NR-Bipy, **10**). The fifth form is a salt with piperazine (PIP^{+}-NR^{-}, **13**).

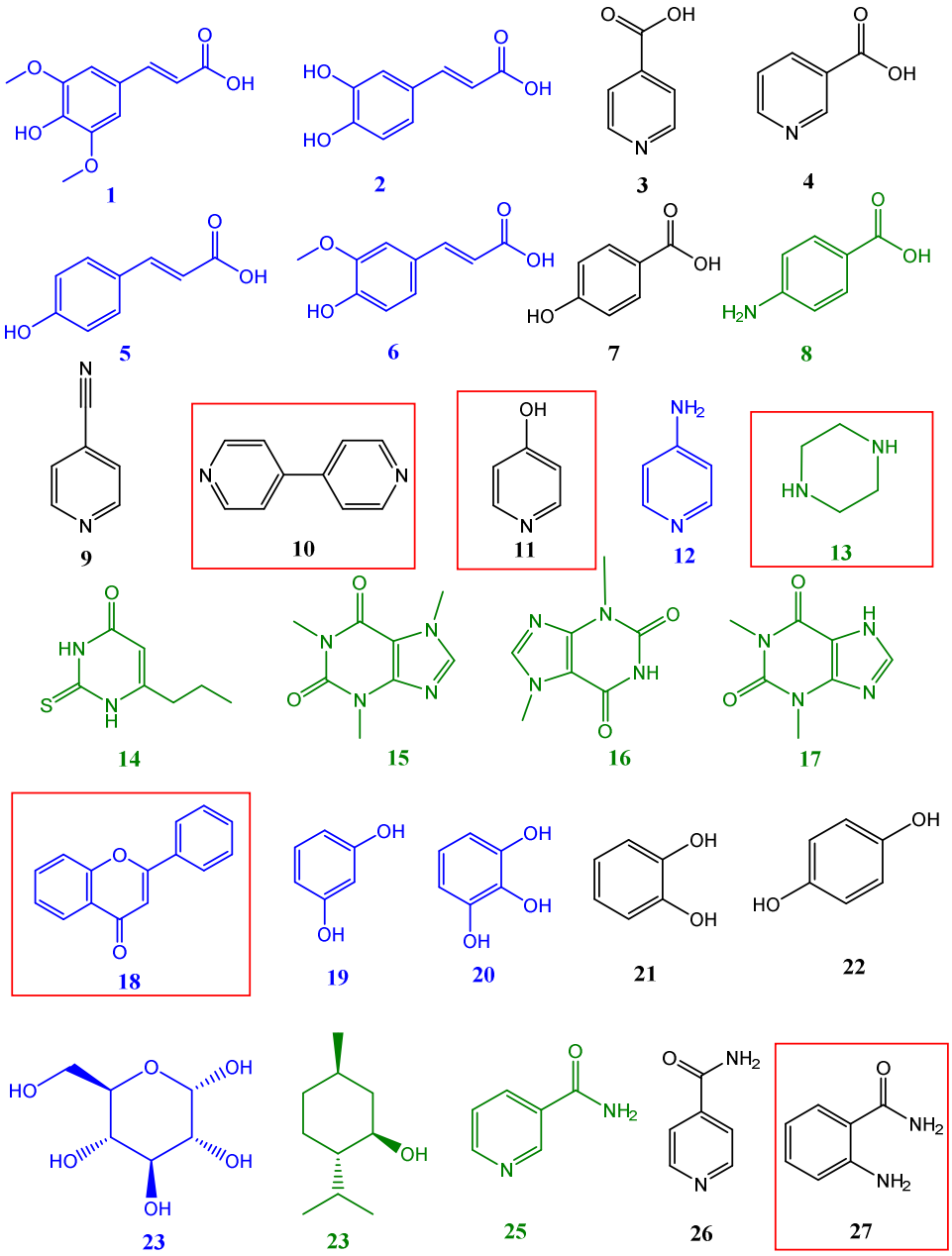


Figure 2 Coformers investigated in this study: nutraceuticals and GRAS coformers (blue), drugs (green), non-GRAS coformers (black), and the coformers which formed cocrystals with NR (red box).

Experimental

Materials

Naringenin was purchased from TCI chemicals, all the coformers and HPLC solvents were purchased from Sigma Aldrich and were used as received without further purification.

Grinding experiments

Mechanical grinding experiments were conducted in a Retsch MM400 Mixer mill, equipped with stainless steel 5 mL grinding jars and one 2.5 mm stainless steel grinding ball per jar. The mill was operated at a rate of 30 Hz for 30 min. The ratio of 1:1 of Naringenin with the coformers was used. In all cases, a powdered material was isolated and analyzed by powder X-ray diffraction (PXRD) and IR (see Supporting Information).

Solution Crystallization

Based on the stoichiometric ratio determined from the neat grinding experiments, the naringenin and the coformer were mixed in the solid state, dissolved in a 1:4 (*v/v*) mixture of methanol and IPA at ~35 °C in a sample vial, covered with perforated parafilm, and left at room temperature until the solvent had completely evaporated, typically 1-2 days. The obtained single crystals were suitable for further analysis by SCXRD.

Physical Measurements

A Bruker Tensor ATR 37 spectrometer was employed to analyse the compounds using OPUS 7.2 (Bruker Optics, Ettlingen, Germany). Samples were placed on diamond probe attenuated total reflectance (ATR) crystal accessory, and 32 scans were collected for each sample at a resolution of 2 cm^{-1} over a wavenumber region of 425-4000 cm^{-1}. Differential scanning calorimetry (DSC) data were collected using a TA Instruments Q1000. Samples (2-6 mg) were crimped in nonhermetic aluminum pans and scanned from 30 to 250 °C at a heating rate of 10 °C min^{-1} under a continuously purged dry nitrogen atmosphere. PXRD data were collected using a D2 phaser diffractometer using Cu $K\alpha_1$ (λ = 1.5406 Å) radiation. Samples were analyzed over a 2θ range of 3.5−45° with increments of 0.05° at a rate of 2° min^{-1}. Data were evaluated using the DIFFRAC.Eva (Bruker AXS Inc.) software. HPLC analysis was performed on an Agilent 1260 Infinity system with a YMC-Pack ODS-A column (250 x 4.6 mm, 5 μm) at 27 °C, eluting with MeCN : H_2O (55:45) at 1 mL^{-1}, UV detection at 290 nm. Samples were filtered through 20 μm filters prior to analysis. Single crystal X-ray data were

collected on a Bruker APEX II DUO diffractometer,[40] using graphite monochromatized Mo Kα (λ = 0.7107 Å) radiation. The CRYSTALS[41] and APEX[42] suite of programmes, incorporating the SHELX suite of programs,[43] were used. The structures were solved using direct methods and refined by full-matrix least-squares on F^2. All non-hydrogen atoms were located and refined with anisotropic thermal parameters. For NR-FLV and NR-BiPy, standard SHELX refinement procedure was used. Structures NR-4HP, NR-ATA and NR^-PIP^+, were refined in CRYSTALS. The hydrogen atoms were located in a Fourier difference map, but those attached to carbon atoms were repositioned geometrically. The hydrogen atoms were initially refined with soft restraints on the bond lengths and angles to regularize their geometry (C-H in the range 0.93-0.98 Å, N-H to value of 0.86 Å, O-H to value of 0.82 Å) and Uiso(H) (in the range 1.2-1.5 times Ueq of the parent atom). Then, the hydrogen positions were refined as riding. Molecular graphics were prepared in Mercury 3.9[44] and Discovery Studio[45]. Some of the structures experienced disorder in the chiral centre of naringenin, in lower or higher extent. The occupancies of the major and minor components were refined in CRYSTALS. To stabilize the refinement and to obtain reasonable ADPs, the disordered atoms' bond lengths and angles (command 'SAME'), anisotropic displacement parameters ('SIMU') and main directions of movement ('DELU') were restrained to be similar. Please see the discussion for details. Crystallographic data are presented in Table 1.

Table 1. Crystallography data for obtained solid forms

Compound	**NR^-PIP^+**	**NR-FLV**	**NR-ATA**	**NR-4HP**	**NR-BiPy**
Formula	$C_{19}H_{22}N_2O_5$	$C_{30}H_{22}O_7$	$C_{22}H_{20}N_2O_6$	$C_{20}H_{17}N_1O_6$	$C_{25}H_{20}N_2O_5$
MW	358.37	494.47	408.40	367.32	428.43
Crystal system	monoclinic	monoclinic	monoclinic	orthorhombic	orthorhombic
Space group, *Z*	$P\,2_1/c$, 4	$P\,2_1/c$, 4	$P\,2_1/c$, 4	$P\,na2_1$, 4	*P bca,* 8
a, Å	9.5718(18)	9.0624(2)	18.0153(5)	8.7644(2)	12.5657(11)
b, Å	18.849(4)	28.7469(6)	4.88020(10)	33.3301(9)	7.8552(8)
c, Å	10.520(2)	9.0586(2)	22.0784(6)	5.9207(2)	42.994(4)
β, °	109.072(7)	97.2740(10)	104.6790(10)	90	90
V, $Å^3$	1793.8(6)	2340.92(9)	1877.74(8)	1729.54(8)	4243.8(7)

Dc gcm^{-3}	1.327	1.400	1.445	1.411	1.341
μ, mm^{-1}	0.800	0.827	0.886	0.880	0.778
2θ range, °	5.424-67.082	5.16-67.20	4.140-67.126	5.218- 66.972	2.06-67.32
T, K	296	296	296	296	296
Total ref.	20437	28481	22652	10463	26071
Unique ref.	3066	4061	3249	2897	3735
Obs. ref., $I > 2\sigma(I)$	3034	3978	3178	2862	3412
# Parameters	318	347	299	302	292
R_1 [$I > 2\sigma(I)$]	0.0446	0.0555	0.0463	0.0345	0.0604
wR_2	0.0971	0.1302	0.1100	0.0792	0.1747
S	1.0808	1.065	0.9732	0.9847	1.066

Solubility experiments:

Slurry experiments were performed for 24 h and the compounds filtered using Whatmann filter paper. The filtrate was then filtered using 0.2 μ filters fitted to a 5 mL syringe for HPLC analysis. HPLC chromatograms were collected and the solid residue was air dried to determine the stability of the solid forms using IR and PXRD. All the slurry experiments and standard curves for selected NR solid forms were tested in 20:80 (v/v) ethanol and water solvent medium at 27 °C.

Results and Discussion:

Five of the 27 investigated coformers successfully formed new multi-component materials with naringenin (NR) as evidenced by IR spectroscopy, DSC and PXRD. Four of the forms are cocrystals; namely naringenin with flavone (NR-FLV), 4-hydroxypyridine (NR-4HP), anthranilamide (NR-ATA) and 4,4′-dipyridyl (NR-Bipy). The fifth form is a salt with piperazine (PIP^+-NR^-). The observed ionization states of these forms correspond to the expected ones based on ΔpKa considerations (see Table S1 in Supporting Information).

All the novel naringenin containing solid forms can be reproduced in the bulk quantities and the PXRDs of these compounds are perfectly matching with the theoretical patterns obtained

from the single crystal X-ray diffraction analysis, indicating the purity and identity of the prepared materials.

Multicomponent solid form screening was carried out by using IR spectroscopy and followed by powder X-ray diffraction (PXRD). In the IR spectroscopy, both blue and red shifts were observed for the novel forms, see Table 2.

Table 2. IR table for NR and its new solid form C=O stretching frequency comparison

NR/solid form	**$\nu_{C=O}$, cm^{-1}**
NR	1625
NR-ATA	1642
NR-Bipy	1614
NR-FLV	1614
NR-4-HP	1627
PIP^{+}-NR^{-}	1635

In the case of the flavone cocrystal, there is a red shift observed from 1625 cm^{-1} to 1614 cm^{-1}, which is expected due to the breaking of strong R_7^7 (14) dimeric units in flavone to form new monomeric chains with the naringenin molecule in its cocrystal form. 4-Hydroxypyridine exists as the 4-(1H)pyridone (tautomeric form of 4-hydroxy pyridine) in its cocrystal and, hence, a red shift in N-H bending region from 1588 cm^{-1} to 1570 cm^{-1} was observed. However, in the case of piperazine salt, due to the single side protonated piperazine, there is a clear blue shift observed in the N-H stretching frequency region from 3215 cm^{-1} to 3335 cm^{-1} and in the lower stretching frequency region 1625 cm^{-1} to 1635 cm^{-1}.

The DSC thermograms have single sharp endotherms, which indicates that the samples are pure (see Supporting Information). There is no evidence for polymorphic transitions. In all cases, the melting point of the multicomponent form was between the naringenin and coformer, Table 3, in agreement with Bak and Stanton's observation of cocrystal melting points.[46]

No strong correlation exists between the melting point of the coformer and the melting points of the naringenin multicomponent solid forms (see Figure S45 in Supporting Information). Such a correlation could be expected in a series of multicomponent forms of a compound,[47] however, it has been shown on the case of methylephedrine salts that these correlations only

exist in isostructural families.[48] This is not the case for these multicomponent forms of naringenin since they are not isostructural (see below).

Table 3. Melting points and enthalpies

Compound name	Single compound, °C	Cocrystal / Salt, °C	ΔH, J g^{-1}
Naringenin	249-252	----	129.9
Anthranilamide	110-113	156-158	93.9*
Piperazine	109-112	181-184	155.8*
Flavone	94-97	192-194	100.8*
4,4′-Bipyridine	111-114	210-213	57.3*
4-Hydroxypyridine	148-152	213-216	92.3*

* value for the NR multicomponent form

Single Crystal X-ray Analysis:

Good quality single crystals were grown from methanol and isopropyl alcohol medium for each of the NR solid forms. The cocrystals of NR with ATA, FLV, and the salt with PIP were solved in monoclinic space group $P2_1/c$, whereas the cocrystals with 4,4'-Bipy and 4-HP were solved in the orthorhombic space groups *Pbca* and $Pna2_1$, respectively. All the novel solid forms have 1:1 naringenin:coformer stoichiometry with one of each of the molecules in the asymmetric unit. None of the structures were solvated or hydrated.

All of the structures are centrosymmetric, which correspond to the racemic character of the material used. Interestingly, some of the structures experience a disorder in the chiral centre, meaning that the structures are in fact racemic solid solutions. The same effect was observed for a recent structural analysis of (±)-naringenin,[38] although an older structural analysis displayed no disorder.[37] Examples of similar anomalous racemates include tetramisole, carvone and trospium chloride.[49-51] For this to be feasible, the molecules of the enantiomers need to have similar shapes and non-covalent interactions to allow for a stable crystal lattice when they are interchanged. For the structures presented in this work, NR-PIP and NR-4HP were modelled in two orientations with the final refined occupancies of 0.57:0.43/0.43:0.57 and 0.51:0.49/0.49:0.51, respectively. In NR-Bipy and NR-ATA, the disorder was a minor component (less than 10 %) that did not allow for a stable refinement of the disorder (the disorder causes strong overlaps in the C ring of naringenin) and therefore, the model presented has no disorder. The flavone cocrystal was not disordered.

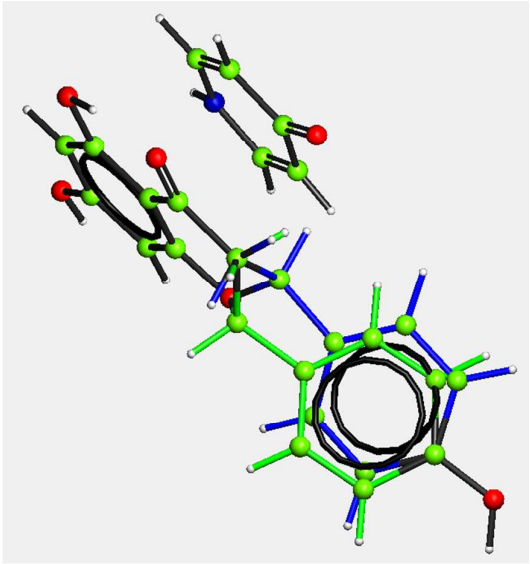


Figure 4. The disorder in the chiral centre of naringenin in NR-4HP.

In the crystal structure of pure naringenin, there is an intramolecular O–H···O=C S(6) hydrogen bond involving the carbonyl oxygen atom of the pyrene ring (B) and the adjacent hydroxyl group (A). Two naringenin molecules hydrogen bond via O–H···O hydrogen bonding involving the remaining hydroxyl group in ring A to form R_2^2 (24) rings, Figure 4. The dimeric rings are hydrogen bonded via O–H···O=C hydrogen bonds involving the hydroxyl group C. Thus the carbonyl oxygen atom is bifurcated. Combination of these hydrogen bonds gives rise to a large R_6^6 (40) ring that contains four naringenin molecules.

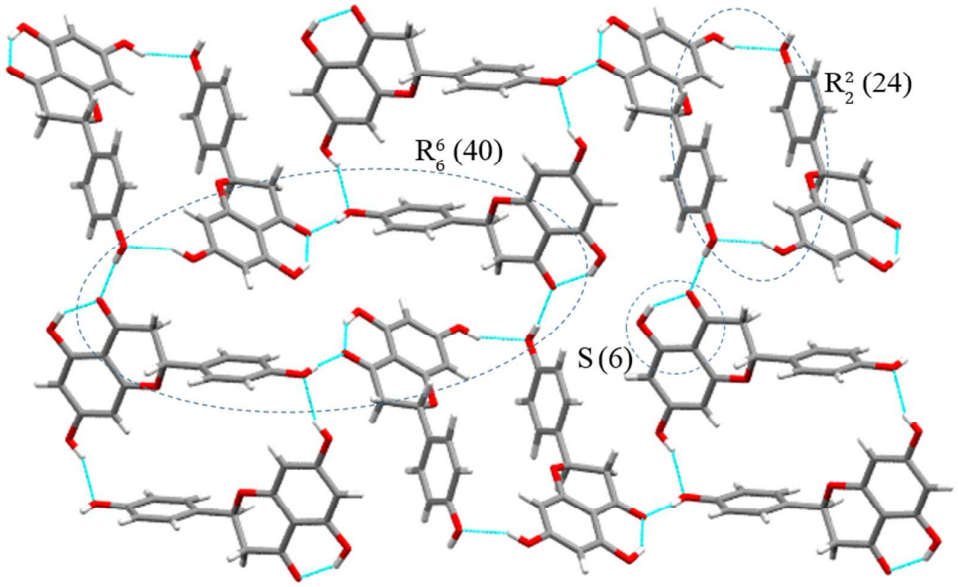
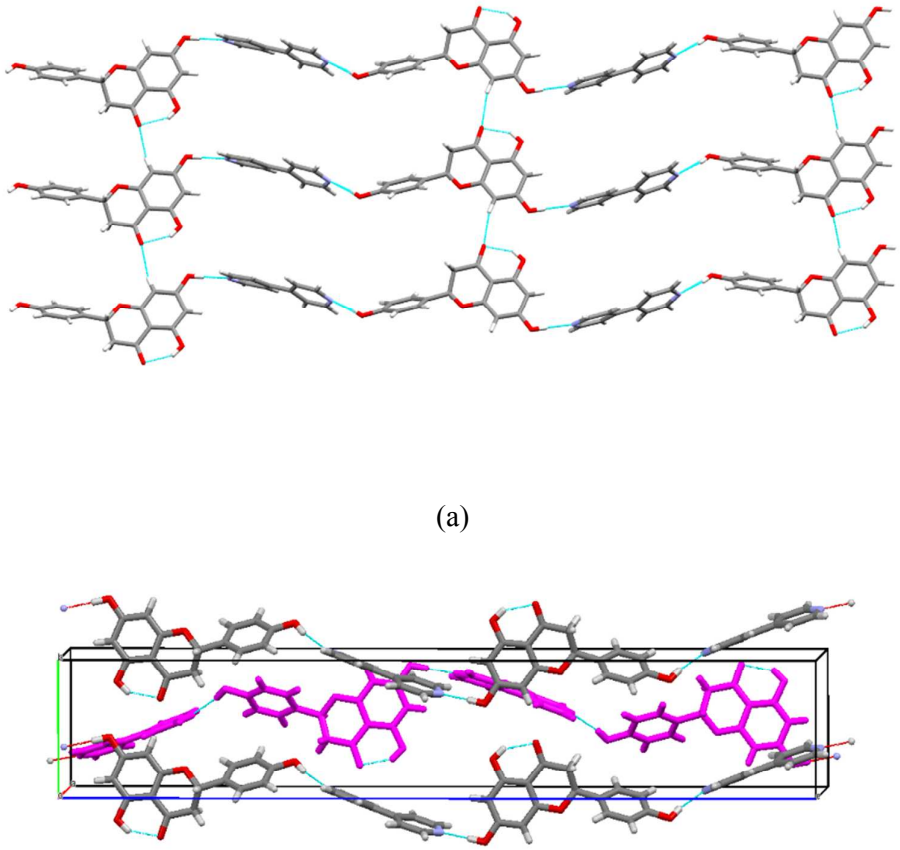


Figure 5. S(6), R_2^2 (24) and R_6^6 (40) hydrogen bonding present in (±)-naringenin[42]

The O–H···O=C S(6) hydrogen bond seen in crystal structure of pure naringenin is preserved in all the multicomponent forms, but the cyclic dimers are not retained in all cases. The overall crystal packing of naringenin is very different in each and every crystal structure, as determined by molecular packing similarity calculation performed in CrystalCMP[52] (See Figure 16 in Supporting Information). For clarity, the disorder has been omitted from the following crystal structures' description.

NR-Bipy (1:1) cocrystal:

In NR-Bipy, the two free phenolic groups on rings A and C form hydrogen bonding network by interacting with both nitrogen atoms on bipyridine via O–H···N hydrogen bonding, giving rise to H-bonded chains. These chains are aligned only along the *c*-axis and interconnected through C–H···O bonds involving the carbonyl oxygen atom to form 2-D buckled layers, Figure 6. This is a commonly seen structural motif in cocrystals of flavonoids containing pyridine based coformers.[53]



(a)

(b)

Figure 6. Crystal structure of NR-Bipy cocrystal; (a) 2D tapes (b) 3D alignment of tapes.

NR-FLV (1:1) cocrystal:

Flavone does not contain any moderate hydrogen-bond donors, meaning that molecules are connected via weak C-H···O=C and CH-π interactions [CSD reference code WADRAV].[54] In contrast, in NR-FLV, flavone molecules are bridging between naringenin dimers through C–H···O and O–H···O=C hydrogen bonding, Figure 7. The R_2^2 (24) ring dimers seen in naringenin are replaced by R_2^2 (20) dimers, utilising the hydroxyl group on ring C and the *keto*-oxygen on pyrone ring B, *via* O–H···O hydrogen boding.

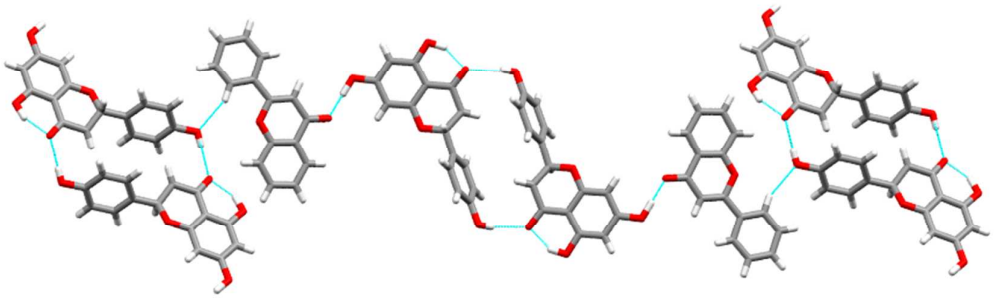
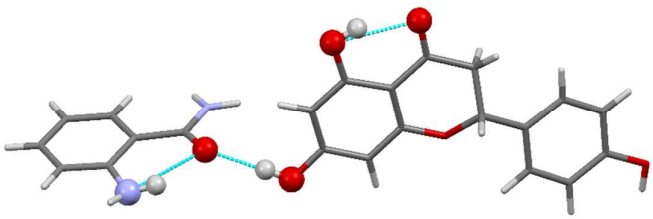


Figure 7. Flavone capped naringenin dimers in their co-crystal

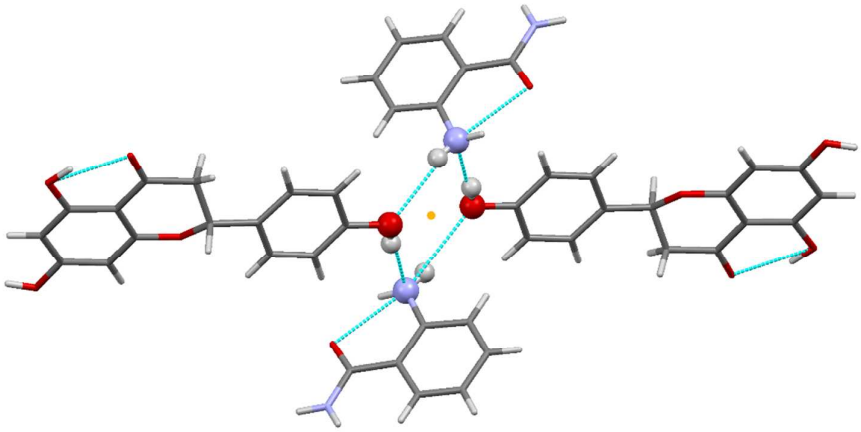
NR-ATA (1:1) cocrystal:

In NR-ATA, one of the hydrogen atoms on the amine functional group is involved in an S(6) intramolecular hydrogen bond with the amide group, whereas this feature hasn't been seen in the reported individual anthranilamide crystal structure [CSD reference code JIXCIC].[55] Thus, only three N–H donors (one from amine and two from amide groups) are available to participate in hydrogen bonding with naringenin.

The amino group with one available N–H forms a centrosymmetric N–H···O, O–H···N hydrogen bonded R_4^1 (8) tetramer with the hydroxyl group present on ring C of naringenin, Figure 8. The other hydroxyl group (A) forms an O–H···O hydrogen bond with the carbonyl oxygen of the amide. The *syn* H atom of the amide is involved in N–H···O hydrogen bond with the hydroxyl oxygen atom of naringenin (A) over the inversion center. The anti H atom of the amide group is interacting via N–H···O hydrogen bonding with the carbonyl group of the naringenin over the screw axis and generates an infinite H-bond chain consisting of (*syn*) N–H···O–H(*intra*)···O···H–N(*anti*), over the 2_1-screw axis along *b* and that generates a H-bond layers parallel to (-1 0 2). The naringenin molecules connecting such hydrogen bond layers in the third dimension are arranged head to tail over the inversion center.



(a)



(b)

Figure 8. Two views of the crystal structure of the naringenin anthranilamide cocrystal showing the hydrogen bonding between the two components.

NR-4HP (1:1) cocrystal:

The crystal structure of NR-4HP reveals that the 4-hydroxypyridine adopts the stable preferred 4-(1H)pyridone tautomer (just one structure found in CSD as 4-hydroxypyridine among 12 hits).[56] The pyridone molecule is interacting with the carbonyl group of naringenin via N–H···O hydrogen bonding via O–H···O hydrogen bonding through its carbonyl oxygen atom with the hydroxyl group on C of naringenin located on the core and extends into an infinite tape, Figure 9. The phenol side arms of naringenin from pairs of such infinite tapes related by a glide plane, interdigitated and connected to carbonyl group of pyridone into a ladder via O–H···O hydrogen bonding. Such ladders are arranged parallel and stacked in offset mode and hence form 2D layers of ladders. The ladders in such stacked layers are connected to ladders in adjacent layers via N–H···O hydrogen bonds to the phenolic oxygen which is involved in intramolecular H-bond to form a 3D structure.

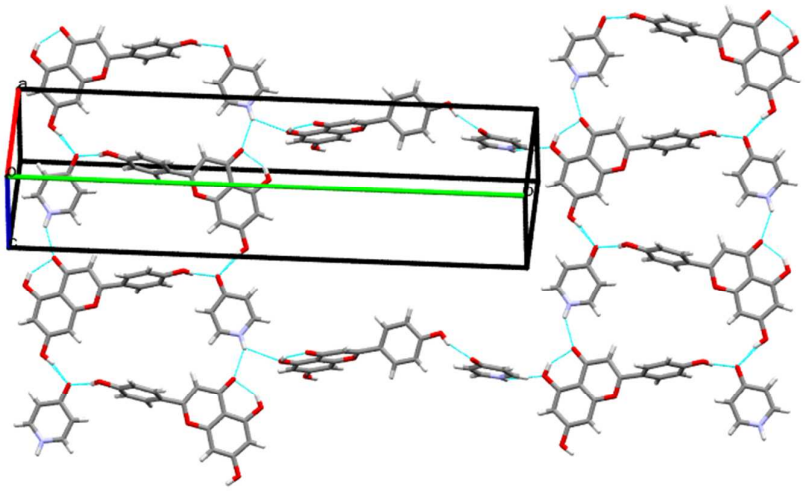
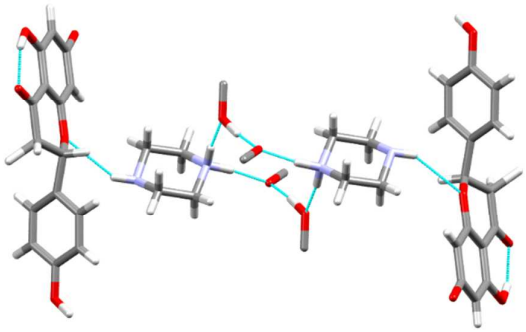


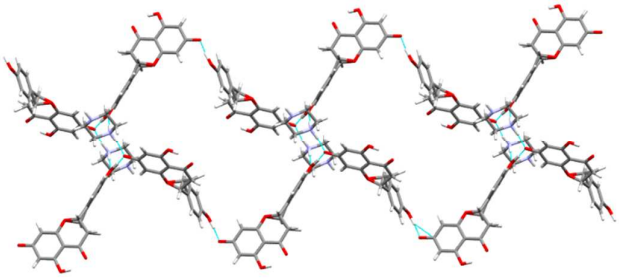
Figure 9. Crystal structure of naringenin: 4-hydroxypyridine (as the 4-pyridone tautomer) showing the ladder formation.

NR^{-} PIP^{+} (1:1) salt:

The piperazinium NH_2^+ group forms a R_6^4 (12) motif involving two deprotonated phenoxides and hydroxyl group on C naringenin. The other N–H group of piperazine forms N–H···O hydrogen bonding with the ether oxygen atom of naringenin, Figure 10.



(a)



(b)

Figure 10. Crystal structure of piperazinium naringeninate. Note that the proton transfer from naringenin to piperazine in the R_6^4 (12) hydrogen bond motif involving (two hydroxyl and two phenoxide groups). The second NH piperazine is hydrogen bonding with the ether oxygen of naringenin.

Solubility Study

Solubility studies were undertaken on the viable solid forms (GRAS) of naringenin, namely the NR-FLV dual nutraceutical cocrystal and the PIP^+-NR^- salt. Due to the poor solubility of parent naringenin in water, these studies were conducted in an ethanol (20%) water (80%) mixture.

As expected, the solubility experiments on $NR^- PIP^+$ salt have shown significant improvement in the solubility (632 mg mL^{-1}) in comparison to naringenin (0.2 mg mL^{-1}). NR-FLV decomposed within the inital five minutes during the equilibrium solubility study as evidenced by IR, HPLC and PXRD data. The PXRD pattern of the dried NR-FLV (residue on the filter paper) does not match with any of the known individual forms or cocrystals and HPLC analysis of the filtrate showed several components (see Supporting Information).

During the review process for this manuscript, Luo et al. described cocrystals of naringenin with betaine, picolinic acid and isonicotinamide.[57] The latter was not discovered in our work, however, their work used a different solvent system to this work, which is most likely responsible for the different results.

Conclusions:

Naringenin was found to form four cocrystals and one salt, all with 1:1 stoichiometry, from the 27 coformers investigated. A number of different H-bonded motifs were adopted in these multi-component forms reflecting the wide variety of hydrogen bond donors and acceptors in the individual components. Some of the structures exhibited disorder in the chiral centre on naringenin, and are, in fact, anomalous racemic solid solutions. The structural analysis reveals that the S(6) O–H···O=C in naringenin is maintained in all five multicomponent forms and that the carbonyl oxygen atom is bifurcated in all four neutral cocrystals. The cocrystal with 4-hydroxypyridine reveals that it adopts the 4-(H)-pyridone tautomer in agreement with all known solid-state structures involving this system and its simple analogues.

ASSOCIATED CONTENT

Supporting Information. IR, PXRD, DSC, HPLC and solubility data, and additional figures. The crystallographic data have been deposited with the Cambridge Crystallographic Data Center, CCDC deposition numbers: 1836185-1836189. The data can be obtained free of charge from the Cambridge Crystallographic Data Center via www.ccdc.cam.ac.uk/data_request/cif. This material is available free of charge via the Internet at http://pubs.acs.org.

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Cocrystals and a Salt of the Bioactive Flavonoid: Naringenin

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Synopsis

Cocrystallization studies on Naringenin with 27 coformers have led to the formation of five new solid forms: piperazinium salt and four cocrystals with the coformers flavone, 4-hydroxypyridine, anthranilamide, and 4,4′-bipyridine. Solubility studies on the pharmaceutically relevant coformers reveal significant enhancement for the piperazinium salt.