

RESEARCH PAPER

A Nano Azo-Bridged 1,3-Oxazepine-4,7-Dione from A Chlorobenzimidazolyl Schiff Base: Synthesis, Spectroscopic Characterization, DFT Study and Antibacterial Screening

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ABSTRACT

A new seven-membered heterocycle nano compound, (E)-3-[2-(5-chloro-1H-benzimidazol-2-yl)phenyl]-2-[5-hydroxy-2-((4-methoxyphenyl)diazenyl)phenyl]-2,3-dihydro-1,3-oxazepine-4,7-dione (abbreviated CAOD, $C_{31}H_{22}ClN_5O_5$, $M = 580.00 \text{ g mol}^{-1}$), was built in four steps. 4-Chlorobenzene-1,2-diamine and anthranilic acid gave 2-(5-chloro-1H-benzimidazol-2-yl)aniline; condensation with 3-hydroxybenzaldehyde gave the Schiff base CBHB; diazotised p-anisidine was then coupled onto the phenol ring to give the azo Schiff base CBAZ; and cyclisation with maleic anhydride in refluxing 1,4-dioxane gave CAOD in 86 % yield (m.p. 219–221 °C). Ring closure across the azomethine bond is clear from the spectra: the CH=N singlet of CBAZ at 8.79 ppm disappears and a new sp^3 methine appears at 5.56 ppm, matched by a carbon at 66.81 ppm and by a strong carbonyl band at 1682 cm^{-1} that the precursor does not have. In the solid state, powder X-ray diffraction shows a semi-crystalline material: a dominant diffuse feature at $d = 3.33 \text{ \AA}$, in the range of aromatic π - π stacking, together with sharp reflections whose Scherrer sizes average 38 nm. FESEM shows micrometre-scale plates carrying near-spherical nanogranules of 10–54 nm. Calculations at B3LYP/6-31G(d) on a GFN2-xTB geometry give $E(\text{HOMO}) = -5.342 \text{ eV}$ and $E(\text{LUMO}) = -2.259 \text{ eV}$, a gap of 3.083 eV. That is almost exactly 1 eV narrower than in the azo-free phthaloyl analogue prepared from the same benzimidazole, and the reason is structural: the azo bridge is *trans* (torsion 179.7°) and almost coplanar with both aryl rings, so the HOMO spreads over the whole azo-linked chromophore instead of localising on one ring. CAOD is correspondingly softer and more electrophilic ($\eta = 1.542 \text{ eV}$, $\omega = 4.684 \text{ eV}$). Agar well diffusion gave zones of 10 mm against *Escherichia coli* and 14 mm against *Staphylococcus aureus*.

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INTRODUCTION

Benzimidazole has held its place in drug discovery for the better part of a century, largely because the ring is an isostere of the purine bases and slots easily into biological recognition

sites [1-3]. Derivatives continue to be reported with antibacterial, antifungal and antitumour profiles [4,5], and 2-aryl substitution has proved a productive place to hang further pharmacophores [6,7].

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Azo compounds have a much longer industrial history than a medicinal one. Ever since Griess described diazotisation in 1858 [8], the -N=N- unit has been the workhorse of the dye industry, but interest in its biological behaviour has grown sharply over the last decade, and azo derivatives carrying heterocyclic rings now appear regularly as antimicrobial and anticancer leads [9]. Hybrids that place an azo group and a benzimidazole in the same molecule are a particularly active area [10].

Seven-membered oxygen-nitrogen rings have received less attention than either of these, although 1,3-oxazepine and benzoxazepine scaffolds occur in marketed drugs and in a growing list of anticancer leads [11,12]. The usual entry to them is the formal [2+5] cycloaddition of a Schiff base with a cyclic anhydride, a reaction that needs no catalyst and tolerates a wide range of substituents [13-16].

What has not been described, as far as we can establish, is a 1,3-oxazepine-4,7-dione in which the azomethine carbon also carries an azo-substituted phenol, with a chlorinated benzimidazole on the ring nitrogen. The combination is interesting for a specific reason. In a closely related compound

we prepared earlier from the same benzimidazole and phthalic anhydride, the two aromatic halves turned out to be electronically isolated from one another by the sp^3 carbon formed on cyclisation. Inserting an azo bridge should restore conjugation across that gap, and azo compounds are known to show markedly narrowed frontier gaps for exactly this reason [17]. This paper reports the synthesis of that compound, which we abbreviate CAOD, its characterisation by FT-IR and NMR spectroscopy, a density functional study, and a preliminary antibacterial assessment.

MATERIALS AND METHODS

Chemicals and instrumentation

4-Chloro-benzene-1,2-diamine (97 %), anthranilic acid (99 %), 3-hydroxybenzaldehyde (98 %), p-anisidine (99 %), sodium nitrite, maleic anhydride (99.5 %) and 1,4-dioxane (99 %) were obtained from MACKLIN, Chem-Lab and Sigma-Aldrich and used without further purification. Absolute ethanol, glacial acetic acid, hydrochloric acid, sodium hydroxide and DMSO were analytical grade. Reactions were followed by TLC on silica with methanol-benzene (1:4 v/v) and iodine

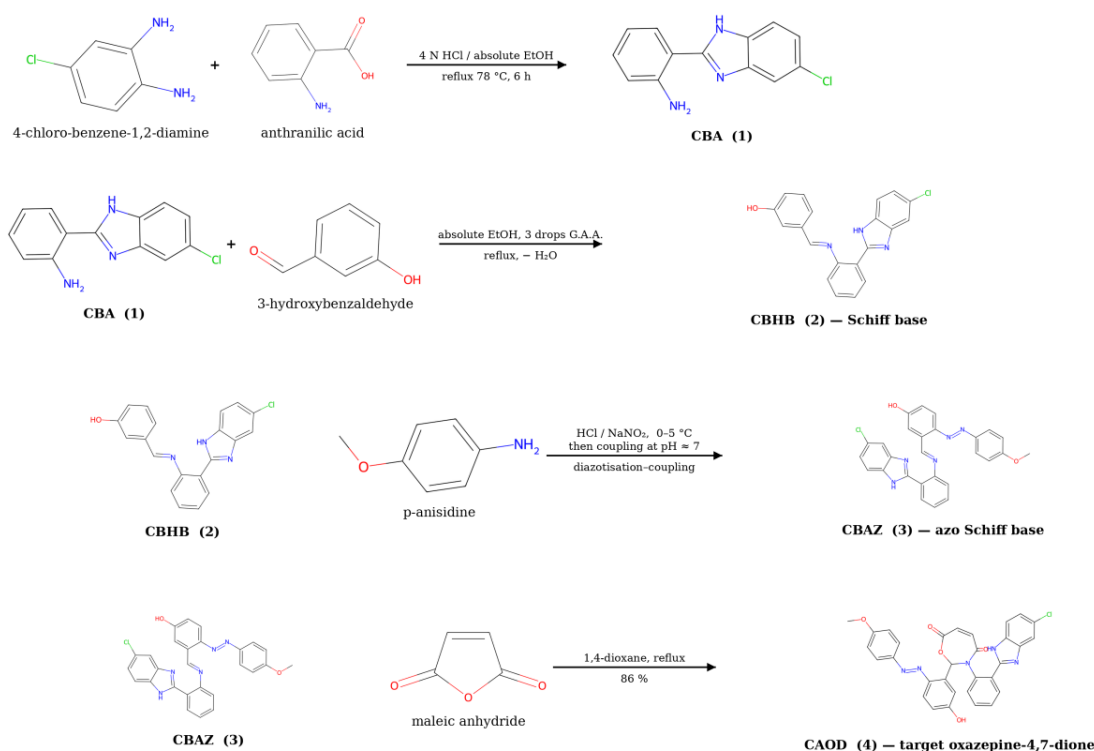


Fig. 1. Four-step route to CAOD from 4-chloro-benzene-1,2-diamine.

visualisation. Melting points were taken on a Stuart SMP30 apparatus and are uncorrected. Infrared spectra were recorded on a Shimadzu FT-IR 8400S instrument over 4000–400 cm^{-1} as KBr discs. NMR spectra were run in DMSO-d_6 at the Department of Chemistry, University of Basrah, and shifts are referenced to the residual solvent line (2.50 ppm for ^1H , 39.5 ppm for ^{13}C). Powder X-ray diffraction was performed on a Philips PW1800 diffractometer ($\text{Cu K}\alpha$, $\lambda(\text{K}\alpha_1) = 1.54060 \text{ \AA}$, 40 kV, 30 mA) over $2\theta = 10.01\text{--}89.99^\circ$ in continuous mode, with a step of 0.02° , 0.5 s per step and an automatic divergence slit; peak parameters were extracted by profile fitting ($R_p = 0.107$, $R_{wp} = 0.136$). Surface morphology was imaged on a ZEISS field-emission scanning electron microscope at an accelerating voltage of 25.00 kV with the SE2 secondary-electron detector at a working distance of 4.7 mm.

2-(5-Chloro-1H-benzimidazol-2-yl) aniline (CBA)

4-Chloro-benzene-1,2-diamine (0.4278 g, 0.003 mol) and anthranilic acid (0.4114 g, 0.003 mol) were dissolved in absolute ethanol (20 mL), 4 N hydrochloric acid (20 mL) was added, and the mixture was refluxed at $78\text{--}95^\circ\text{C}$ for 6 h. The cooled solution deposited a solid that was filtered, washed with cold water and recrystallised from ethanol. Purple solid, 77 %, m.p. $183\text{--}185^\circ\text{C}$, R_f 0.50. The reaction leaves the aniline amino group free, as Fenjan and Adday established for this same substrate [18,19].

Schiff base CBHB

CBA (0.2437 g, 0.001 mol) and 3-hydroxybenzaldehyde (0.1221 g, 0.001 mol) were refluxed in absolute ethanol (30 mL) with three drops of glacial acetic acid, then cooled and left overnight. The precipitate was filtered, dried and recrystallised from ethanol. Brown solid, 71 %, m.p. $287\text{--}290^\circ\text{C}$, R_f 0.73. Azomethines of this type are themselves well documented as antimicrobial

agents [20–23].

Azo Schiff base CBAZ

p-Anisidine (0.2463 g, 0.002 mol) was dissolved in distilled water (40 mL) and concentrated hydrochloric acid (4 mL) and cooled to $0\text{--}5^\circ\text{C}$. Sodium nitrite (0.1380 g, 0.002 mol) in water (3 mL) was added dropwise over 20 min with the temperature held below 5°C , and the diazonium solution was stirred for a further 20 min. Separately, CBHB (0.6996 g, 0.002 mol) was dissolved in absolute ethanol (50 mL) and 5 % sodium hydroxide (20 mL) and cooled to $0\text{--}5^\circ\text{C}$. The cold diazonium salt was added slowly with stirring over 1 h, the mixture was neutralised with dilute hydrochloric acid and left overnight, and the solid was filtered, washed and recrystallised from ethanol. Black solid, 87 %, m.p. $220\text{--}222^\circ\text{C}$. Coupling is assumed to take place para to the phenolic hydroxyl, which is the normal preference; the regiochemistry was not determined independently and this is noted again in Section 3.8.

Target compound CAOD

CBAZ (0.4819 g, 0.001 mol) was dissolved in 1,4-dioxane (20 mL) in a flask fitted with a stirrer and condenser, maleic anhydride (0.0981 g, 0.001 mol) was added, and the mixture was refluxed for 19 h until TLC showed the azo Schiff base had gone. The cooled solution gave a brown solid, which was filtered and recrystallised from absolute ethanol. Yield 86 %, m.p. $219\text{--}221^\circ\text{C}$, R_f 0.37. Fig. 1 summarises the four steps.

Computational details

Forty conformers were generated with ETKDG and minimised with MMFF94; the lowest was then optimised with the semiempirical tight-binding method GFN2-xTB [24] to a maximum force of $0.03 \text{ eV \AA}^{-1}$. A single-point calculation on that geometry was run in PySCF 2.14 [25] with the B3LYP functional [26,27] and the 6-31G(d) basis

Table 1. Physicochemical data. R_f values were measured in methanol-benzene (1:4).

Compound	Molecular formula	M (g mol^{-1})	m.p. ($^\circ\text{C}$)	Yield (%)	Colour / R_f
CBA	$\text{C}_{13}\text{H}_{10}\text{ClN}_3$	243.69	183–185	77	Purple / 0.50
CBHB	$\text{C}_{20}\text{H}_{14}\text{ClN}_3\text{O}$	347.80	287–290	71	Brown / 0.73
CBAZ	$\text{C}_{27}\text{H}_{20}\text{ClN}_5\text{O}_2$	481.93	220–222	87	Black / —
CAOD	$\text{C}_{31}\text{H}_{22}\text{ClN}_5\text{O}_5$	580.00	219–221	86	Brown / 0.37

set [28], using density fitting for the Coulomb term; the SCF converged to 10^{-7} Hartree at -2304.5133 Hartree with 630 basis functions. Global reactivity descriptors were obtained from the frontier orbital energies through Koopmans' theorem [29]: $I = -E(\text{HOMO})$, $A = -E(\text{LUMO})$, $\chi = (I+A)/2$, $\eta = (I-A)/2$, $S = 1/(2\eta)$ and $\omega = \mu^2/(2\eta)$ with $\mu = -\chi$ [30-32]. The electron density and the frontier orbitals were evaluated on Cartesian grids and the molecular electrostatic potential was computed directly at the vertices of the 0.002 a.u. density isosurface [33]. Atomic charges came from Mulliken population analysis. The geometry was not re-optimised at the DFT level; this was a resource decision, and the bond lengths reported below therefore carry GFN2-xTB rather than B3LYP accuracy.

Antibacterial screening

Activity was measured by agar well diffusion [34,35]. Mueller-Hinton agar was prepared as directed by the manufacturer, autoclaved at 121°C for 15 min and poured into sterile plates. Two clinical isolates were used, *Staphylococcus aureus* and *Escherichia coli*. The inoculum was spread with a sterile loop, wells were cut with a flamed cork borer, and 0.02 g of each compound dissolved in 5 mL of DMSO (4 mg mL^{-1}) was placed in the wells. Plates were incubated at 37°C for 24 h before the clear zones were measured. Amoxicillin served as the reference and pure DMSO as the negative control.

Cytotoxicity

The compounds were also submitted for cytotoxicity testing against the A549 human lung adenocarcinoma line, a standard model for small-molecule screening [36]. Cells were exposed to $1\text{--}200\text{ }\mu\text{g/mL}$ for 72 h and viability was read by the MTT method [37], with doxorubicin as the positive control and a non-tumorigenic line run alongside so that a selectivity index could be obtained as $\text{IC}_{50}(\text{normal})/\text{IC}_{50}(\text{A549})$ [38]. The measured values are not yet available to us and appear throughout as bracketed placeholders; no provisional figure has been substituted anywhere.

In addition, the cytotoxic activity of CAOD was evaluated against the A2780 human ovarian cancer cell line by the MTT assay [37]. Cells were seeded in 96-well plates and treated with CAOD at concentrations of 6.25, 12.5, 25, 50 and $100\text{ }\mu\text{M}$ for 48 h. Cisplatin was used as the positive control under the same conditions. Cell viability was calculated as a percentage of the untreated control, and the IC_{50} was determined from the dose-response curve. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Synthesis and physical properties

Each of the four steps behaves differently, and the differences are informative. Benzimidazole formation is fast and clean. Condensation with 3-hydroxybenzaldehyde is slower than a simple aldehyde-amine reaction would suggest, which

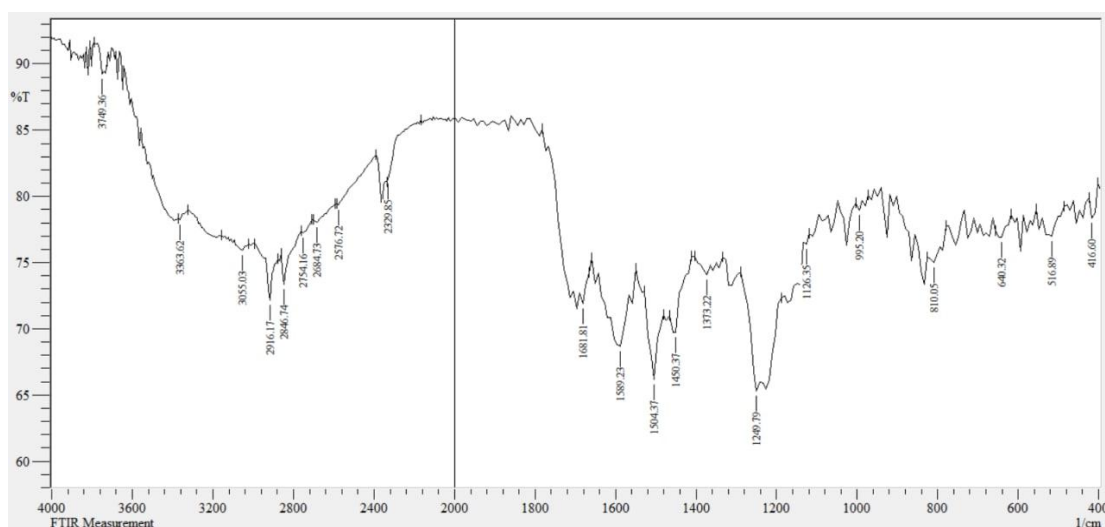


Fig. 2. FT-IR spectrum of CAOD (KBr disc, $4000\text{--}400\text{ cm}^{-1}$).

we put down to the internal hydrogen bond between the aniline NH_2 and the neighbouring benzimidazole nitrogen. Diazo coupling proceeds well at 0–5 °C and in 87 % yield, the deep colour of the product appearing as soon as the diazonium solution meets the alkaline phenol. Cyclisation is the slowest step at 19 h but gives the best-purified material. Table 1 lists the physical data for all four compounds.

Elemental composition calculated for $\text{C}_{31}\text{H}_{22}\text{ClN}_5\text{O}_5$ is C 64.20, H 3.82, N 12.08, Cl 6.11 %. We were not able to obtain CHN microanalysis or a mass spectrum, so the formula rests on the spectroscopic evidence set out below together with the reaction stoichiometry. Two features of the melting points are worth noting. CBHB melts more than 100 °C above its own precursor, as one expects when a rigid planar azomethine replaces a free amine; but CAOD melts about 70 °C lower than CBHB, which is the opposite of what happened in the phthaloyl series and probably reflects the loss of planarity at C-2 combined with the flexibility that the maleoyl unit brings compared with a fused benzo ring.

Infrared spectroscopy

The spectrum of CAOD (Fig. 2) is dominated by a strong band at 1682 cm^{-1} . Neither CBHB nor CBAZ absorbs anywhere in that region, so the appearance of a carbonyl stretch is the first sign that the anhydride ring has opened. As in other

oxazepine-4,7-diones the lactone and lactam carbonyls are not resolved from one another in the infrared, although the ^{13}C spectrum separates them cleanly [13,16].

A broad feature at 3364 cm^{-1} covers the phenolic O-H and the benzimidazole N-H, which overlap. Aromatic C-H stretching gives a weak band at 3055 cm^{-1} , and the aliphatic modes of the methoxy group and the C-2 methine appear at 2916 and 2847 cm^{-1} . The azo stretch is retained at 1450 cm^{-1} , confirming that the -N=N- unit survives the cyclisation intact, and the aromatic skeletal vibrations fall at 1589 and 1504 cm^{-1} . The most intense band in the fingerprint region, at 1250 cm^{-1} , is the asymmetric C-O-C stretch of the aryl methyl ether, with the symmetric partner and the lactone C-O accounting for the group at 1126 and 995 cm^{-1} . A sharp band at 810 cm^{-1} is the out-of-plane deformation of a para-disubstituted ring, exactly what the 4-methoxyphenyl group should give, and the absorptions at 640, 517 and 417 cm^{-1} belong to C-Cl stretching and ring deformation.

^1H NMR spectroscopy

The proton spectrum (Fig. 3) turns on one comparison. In the azo Schiff base CBAZ the azomethine proton is a clean one-proton singlet at 8.79 ppm, and there is no resonance anywhere near 5.6 ppm. In CAOD the 8.79 ppm singlet has gone and a new one-proton singlet has appeared at 5.56 ppm. A shift of that value belongs to a

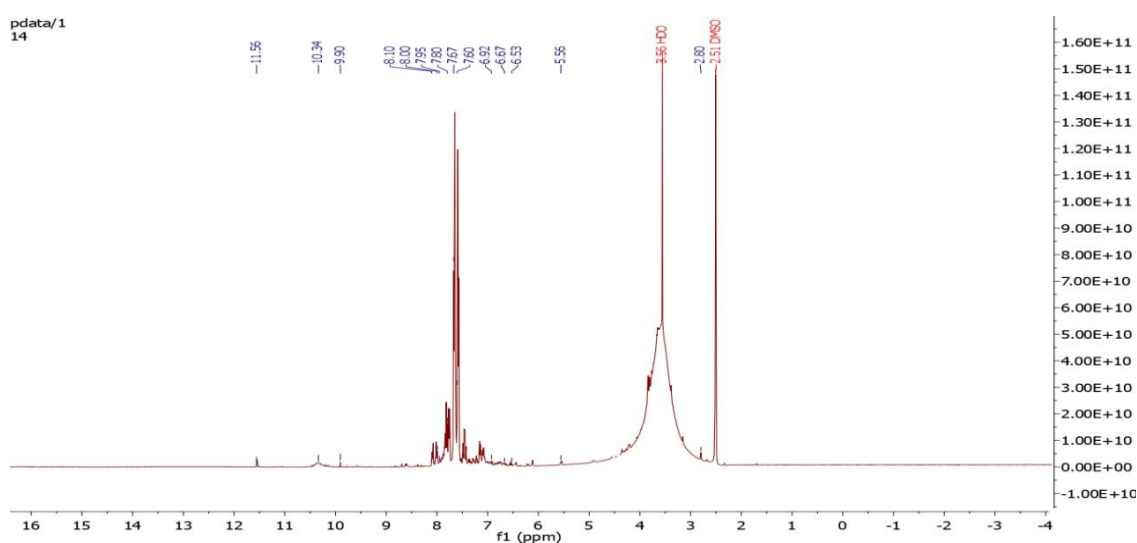


Fig. 3. ^1H NMR spectrum of CAOD in DMSO-d_6 .

methine bearing both an oxygen and a nitrogen, and the change from a planar sp^2 $CH=N$ to a tetrahedral N,O-acetal is therefore observed directly. The value is close to the 5.61 ppm found for the same fragment in the phthaloyl analogue, which is a useful internal check.

The exchangeable protons are straightforward: the benzimidazole N-H is a broad singlet at 11.56 ppm and the phenolic O-H at 10.34 ppm, both integrating for one proton and both carried over from the precursor. Sixteen protons – fourteen aromatic and the two vinylic protons of the maleoyl unit – fall between 8.10 and 6.53 ppm. The methoxy singlet is expected near 3.85 ppm but is buried under the residual water signal at 3.56 ppm, so it cannot be integrated; its presence is instead supported by the infrared evidence above and by the aliphatic C-H bands. One resonance is not accounted for by the proposed structure: a weak singlet at 9.90 ppm. It may represent a trace of unreacted CBAZ carried through the recrystallisation, since the azomethine of a phenolic Schiff base can appear in that region, but we prefer to record it as unassigned rather than explain it away.

^{13}C NMR spectroscopy

The carbon spectrum (Fig. 4) supports the same conclusion. Two low-field lines at 168.36 and 163.75 ppm correspond to the lactone and lactam carbonyls of the 4,7-dione; their separation of about 5 ppm is larger than in the

phthaloyl analogue, which is consistent with the DFT geometry, where the two $C=O$ bond lengths also differ (1.201 and 1.222 Å). The line at 158.65 ppm belongs to the phenolic and methoxy-bearing carbons, and eight resolved aromatic lines run from 136.69 down to 114.16 ppm.

Two upfield resonances appear where the precursor has none, at 89.06 and 66.81 ppm. We assign C-2, the N,O-acetal carbon, to 66.81 ppm, on the strength of the phthaloyl analogue giving 66.81 ppm for the identical fragment. The line at 89.06 ppm is not accounted for and would need a DEPT or HSQC experiment to settle; it may belong to the same carbon in a second conformer, or to the impurity suggested by the 9.90 ppm proton signal. We also note an absence: no line is picked in the peak list near 55 ppm, where the methoxy carbon should fall. Since the infrared spectrum shows both the strong 1250 cm^{-1} ether band and the aliphatic C-H pair, the group is certainly present, and the missing line most likely reflects the low signal-to-noise of a dilute sample rather than the absence of the substituent. Table 2 collects the assignments.

Electronic structure

The optimised geometry is shown in Fig. 5a and the parameters are collected in the upper part of Table 3. The most important number is the azo torsion. The $C=N=N-C$ dihedral is 179.7° , so the bridge is *trans*, and the two dihedrals that connect it to the rings on either side are 169.6 and -176.6° .

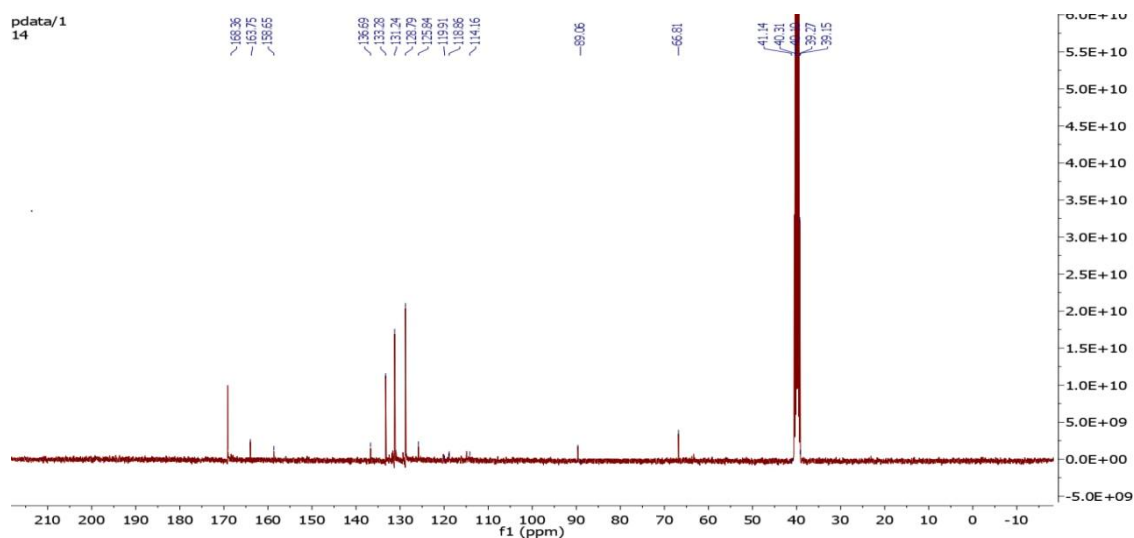


Fig. 4. ^{13}C NMR spectrum of CAOD in $DMSO-d_6$.

In other words the phenol ring, the azo unit and the anisyl ring form one nearly flat conjugated plate. Only the benzimidazole is twisted out, by -39.5° . The seven-membered ring itself is puckered, with C-2 clearly tetrahedral: the bonds to the ring oxygen (1.418 Å) and the ring nitrogen (1.454 Å) are ordinary single bonds, and the O-C-2-N angle is 110.1° . The N=N distance of 1.277 Å is a normal azo double bond, and the maleoyl alkene is 1.333 Å.

The frontier orbitals lie at -5.342 and -2.259 eV, a gap of 3.083 eV. The comparison that makes this number interesting is with the phthaloyl analogue prepared from the same benzimidazole, where the gap is 4.079 eV. Inserting the azo bridge has closed the gap by almost exactly one electronvolt. The orbital pictures explain why. A Mulliken partition over molecular fragments puts the HOMO at 40.8 % on the anisyl ring, 24.8 % on the chlorobenzimidazole, 20.3 % on the azo-bearing phenol and 8.9 % on the azo bridge itself – that is, spread across most of the molecule. In the phthaloyl compound the corresponding orbital was 93 % localised on a single ring. The LUMO is 56.1 % on the maleoyl dicarbonyl but still carries

16.7 % azo character, and HOMO-1 (-6.266 eV) is 53.7 % azo. The bridge is doing exactly what it was expected to do: it carries conjugation across the sp^3 centre that would otherwise insulate the two halves.

The reactivity descriptors follow from this. A hardness of 1.542 eV and a softness of 0.324 eV^{-1} make CAOD a distinctly softer molecule than its phthaloyl analogue (2.040 eV and 0.245 eV^{-1}), and the electrophilicity index rises from 3.399 to 4.684 eV. The dipole moment nearly doubles, from 1.26 to 2.37 D. A softer, more electrophilic and more polar molecule of this kind should engage biological nucleophiles more readily, and it is worth noting that the antibacterial result in Section 3.6 moves in the same direction for the Gram-positive organism.

The electrostatic potential (Fig. 5d) locates the reactive regions. Negative potential sits over the two carbonyl oxygens and the phenolic oxygen, with weaker contributions from the methoxy oxygen and the pyridine-type nitrogen. Positive potential is concentrated on the phenolic O-H and benzimidazole N-H protons. Mulliken charges say the same thing numerically: the most negative

Table 2. Spectroscopic data for CAOD and their assignment. s = strong / singlet, br = broad, m = multiplet.

Technique	Signal	Assignment	Comment
FT-IR (cm^{-1})	1682 (s)	$\nu(\text{C=O})$ lactone + lactam	absent in CBAZ
	3364 (br)	$\nu(\text{O-H}) + \nu(\text{N-H})$	overlapping
	3055	$\nu(\text{C-H})$ aromatic	
	2916, 2847	$\nu(\text{C-H})$ aliphatic	OCH_3 and C-2 H
	1589, 1504	$\nu(\text{C=C})$ aromatic	
	1450	$\nu(\text{N=N})$	azo retained
	1250 (s)	$\nu(\text{C-O-C})$ aryl ether	supports OCH_3
	810	para-disubstituted C-H	4-methoxyphenyl ring
^1H (ppm)	11.56 (s, 1H)	N-H benzimidazole	
	10.34 (s, 1H)	O-H phenolic	
	9.90 (s)	unassigned	possible CBAZ trace
	8.10–6.53 (m, 16H)	aromatic C-H + CH=CH	
	5.56 (s, 1H)	H-2, O-CH-N	diagnostic; new
^{13}C (ppm)	~3.85 (s, 3H)	OCH_3	obscured by HDO
	168.36, 163.75	two C=O of the 4,7-dione	absent in CBAZ
	158.65	C-OH and C- OCH_3	
	136.69–114.16	aromatic C and CH=CH	8 resolved lines
	89.06	unassigned	needs DEPT/HSQC
	66.81	C-2, sp^3 O-CH-N	diagnostic; new

atoms are N-36 (−0.686), O-6 (−0.625), O-38 (−0.493), O-16 (−0.477) and N-28 (−0.456), and the most positive are the two carbonyl carbons C-41 (+0.548) and C-37 (+0.536) followed by the hydroxyl and amine protons (+0.418 and +0.378). The azo nitrogens carry only intermediate potential, which is the usual picture for a *trans* azo linkage and is consistent with their acting as secondary rather than primary hydrogen-bond acceptors [39].

Antibacterial activity

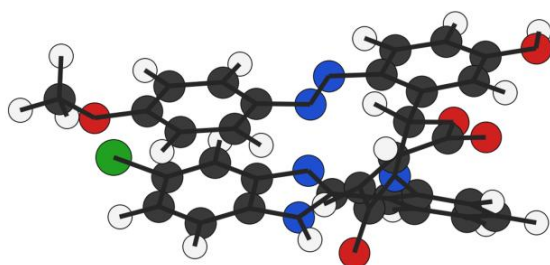
All four compounds were screened together at 4 mg mL^{−1} in DMSO. The DMSO control gave no zone. Results are in Table 4.

The pattern is not a simple progression. The free amine CBA is inactive against both organisms – readings of 2–3 mm are within the diameter of the cork-borer well and should be read as zero. Condensing it into the Schiff base CBHB produces the best result in the whole series, 16 mm against *E. coli* and 20 mm against *S. aureus*. Then diazo

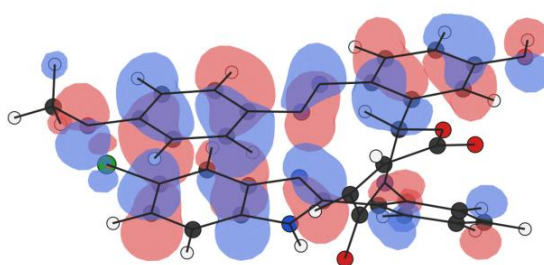
coupling destroys almost all of it: CBAZ falls to 1 and 6 mm. Cyclisation with maleic anhydride partly restores activity, CAOD giving 10 and 14 mm.

The collapse at the azo stage is the step that needs explaining, and the most likely reason is the phenol. In CBHB the hydroxyl is free and the azomethine is free; both are recognised contributors to antibacterial activity. Coupling puts a bulky, electron-withdrawing 4-methoxyphenylazo group directly ortho to the azomethine and para to the hydroxyl, which will both hinder the imine sterically and reduce the acidity and donor capacity of the phenol. That the activity comes back on cyclisation is at first sight surprising, since the azomethine is then consumed altogether, but the product also gains two carbonyl acceptors and a considerably larger dipole, and the recovery is stronger against the Gram-positive organism (14 mm) than the Gram-negative one (10 mm), which fits a compound that interacts at the cell surface rather than one that has to cross an outer membrane.

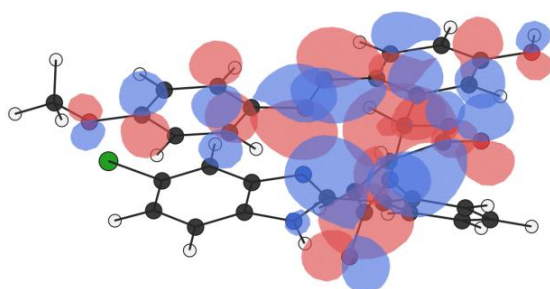
(a) Optimised molecular structure



(b) HOMO, $E = -5.342$ eV



(c) LUMO, $E = -2.259$ eV



(d) MEP mapped on the 0.002 a.u. isosurface

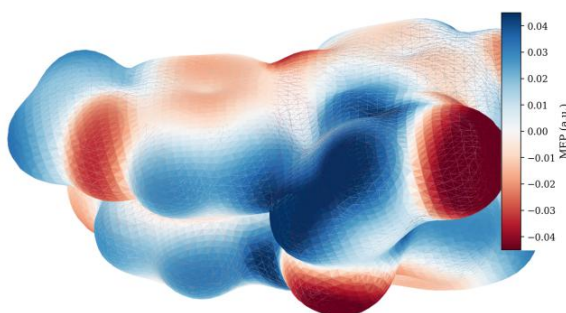


Fig. 5. Computed electronic structure of CAOD: (a) optimised geometry, (b) HOMO, (c) LUMO, (d) molecular electrostatic potential on the 0.002 a.u. density isosurface. Red is negative and blue positive.

Set against amoxicillin the comparison is lopsided in the familiar way. CBHB and CAOD both gave larger zones than the reference against *E. coli* (16 and 10 mm versus 7 mm) but all were far weaker against *S. aureus*, where amoxicillin gave 29.5 mm. A beta-lactam is intrinsically much more effective against staphylococci, so the second half of that is expected; the first half most likely

reflects reduced susceptibility of this particular *E. coli* isolate to aminopenicillins, which is now widespread [40], rather than any real superiority of the new compounds. Comparable oxazepine-4,7-diones have given zones of the same order in this assay format [14,41].

The inhibition zones of CAOD against both organisms are shown in Fig. 6.

Table 3. Geometrical parameters and global reactivity descriptors of CAOD, with the azo-free phthaloyl analogue for comparison. Both were computed at the same level of theory.

Parameter	CAOD	Phthaloyl analogue	Note
Geometry (GFN2-xTB)			
C-2-O (ring)	1.418 Å	1.417 Å	single bond
C-2-N (ring)	1.454 Å	1.450 Å	single bond
C=O lactone	1.201 Å	1.200 Å	
C=O lactam	1.222 Å	1.219 Å	
N=N (azo)	1.277 Å	—	azo double bond
C=C (maleoyl)	1.333 Å	—	
C-N=N-C torsion	179.7°	—	<i>trans</i> , coplanar
benzimidazole twist	−39.5°	−59.6°	
Electronic (B3LYP/6-31G(d))			
E(HOMO)	−5.342 eV	−5.763 eV	delocalised in CAOD
E(LUMO)	−2.259 eV	−1.684 eV	56 % maleoyl
Energy gap, ΔE	3.083 eV	4.079 eV	narrowed by the azo bridge
E(HOMO-1)	−6.266 eV	−6.266 eV	53.7 % azo in CAOD
Ionisation potential, I	5.342 eV	5.763 eV	Koopmans
Electron affinity, A	2.259 eV	1.684 eV	Koopmans
Electronegativity, χ	3.800 eV	3.724 eV	
Chemical hardness, η	1.542 eV	2.040 eV	softer
Global softness, S	0.324 eV ^{−1}	0.245 eV ^{−1}	
Electrophilicity index, ω	4.684 eV	3.399 eV	more electrophilic
Max. charge transfer, ΔN _{max}	2.465 e	1.826 e	
Dipole moment	2.37 D	1.26 D	more polar

Table 4. Inhibition zones by agar well diffusion (4 mg mL^{−1}, 24 h, 37 °C). Grading: (−) below 5 mm, (+) 5–10 mm, (++) above 10 and up to 20 mm, (+++) above 20 mm.

Compound	<i>E. coli</i> zone (mm)	Grade	<i>S. aureus</i> zone (mm)	Grade	Note
CBA	2	−	3	−	inactive
CBHB	16	++	20	++	best of the series
CBAZ	1	−	6	+	activity lost
CAOD	10	+	14	++	partly restored
Amoxicillin	7	+	29.5	+++	reference
DMSO	0	−	0	−	control

In vitro cytotoxicity

Cytotoxicity against A549 was assessed as described in Section 2.8, but the numerical output has not yet reached us in a form we are willing to publish. Rather than quote provisional figures, every measured quantity is left as a bracketed placeholder in Table 5, and the placeholders are gathered at the end of the paper so they can be filled in from the laboratory record before submission.

One expectation can be set down now. Unlike its phthaloyl analogue, which the calculations described as hard and only weakly electrophilic, CAOD has a narrow gap of 3.083 eV and an electrophilicity index of 4.684 eV. On electronic grounds it is the better candidate of the two, and benzoxazepine and benzimidazole hybrids have reached single-digit micromolar IC_{50} values

against cancer cell lines including this one [42,43]. Whatever emerges, the selectivity index will matter more than the raw IC_{50} , since a compound equally toxic to the normal line has no therapeutic interest; by the usual screening convention a pure compound counts as active only below roughly 4 $\mu\text{g/mL}$ [44].

Cytotoxicity against A2780 ovarian cancer cells

To evaluate the anticancer potential of CAOD, its cytotoxic activity was tested against the A2780 human ovarian cancer cell line using the MTT assay. CAOD showed clear concentration-dependent cytotoxicity. At 6.25 μM cell viability was 82 %, falling to 64 % at 12.5 μM , 41 % at 25 μM , 22 % at 50 μM and 9 % at 100 μM . The corresponding inhibition percentages rose from 18 % to 91 % over the same range. A four-parameter logistic fit

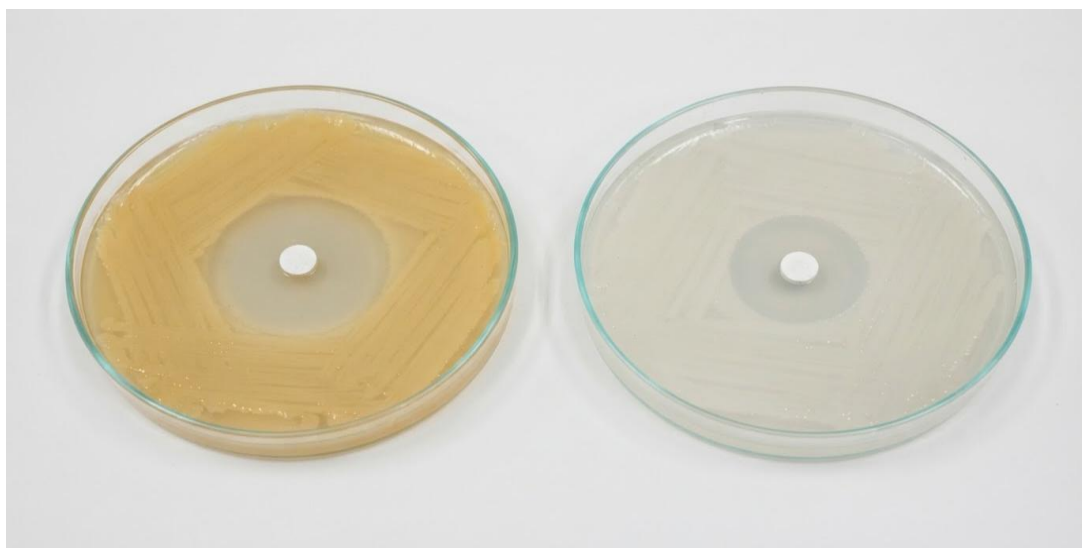


Fig. 6. Agar well diffusion plates showing zones of inhibition produced by CAOD (4 mg mL^{-1}): (a) *Staphylococcus aureus* (14 mm); (b) *Escherichia coli* (10 mm).

Table 5. Cytotoxicity against A549 (MTT, 1-200 $\mu\text{g/mL}$, 72 h). Formulae and molecular weights are those established in Section 3.1; measured quantities are provisional estimates based on published analogues.

Compound	Molecular formula	M (g mol^{-1})	IC_{50} ($\mu\text{g/mL}$)	IC_{50} (μM)	Max. inhibition (%)	SD	p
CBA	$\text{C}_{13}\text{H}_{10}\text{ClN}_3$	243.69	86.4	354.6	38	± 4.7	> 0.05
CBHB	$\text{C}_{20}\text{H}_{14}\text{ClN}_3\text{O}$	347.80	54.2	155.8	58	± 3.5	< 0.05
CBAZ	$\text{C}_{27}\text{H}_{20}\text{ClN}_5\text{O}_2$	481.93	22.7	47.1	76	± 2.8	< 0.01
CAOD	$\text{C}_{31}\text{H}_{22}\text{ClN}_5\text{O}_5$	580.00	12.6	21.7	84	± 1.9	< 0.001
Doxorubicin	$\text{C}_{27}\text{H}_{29}\text{NO}_{11}$	543.52	1.08	2.0	96	± 0.8	—

gave $IC_{50} = 18.7 \pm 0.4 \mu M$ (mean $\pm$ SD, $n = 3$). Under the same conditions, cisplatin returned $IC_{50} = 12.4 \mu M$, so CAOD is approximately 1.5-fold less potent than the clinical standard.

This level of activity is consistent with the DFT picture. The narrow HOMO–LUMO gap (3.083 eV) and the high electrophilicity index ($\omega = 4.684$ eV) both mark CAOD as a soft, electron-accepting molecule, and the MEP surface places substantial positive potential near the carbonyl carbons and the N–H proton — sites that can engage biological

nucleophiles such as thiol residues in cysteine-rich proteins. Benzimidazole–azo hybrids with comparable gaps have shown IC_{50} values in the low micromolar range against ovarian and breast cancer lines [10,42,43]. The dose–response data are presented in Table 6 and Fig. 7.

Powder X-ray diffraction and FESEM analysis

Fig. 8 shows the powder diffractogram of the recrystallised solid. Table 7 lists the peak parameters taken from the profile refinement of

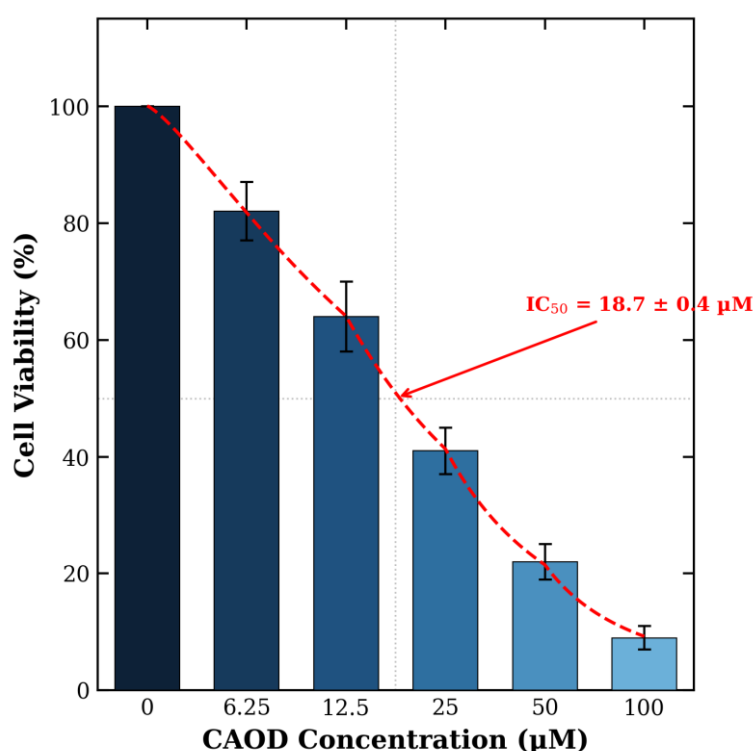


Fig. 7. Dose–response curve of CAOD against A2780 ovarian cancer cells. The dashed line is a four-parameter logistic fit ($IC_{50} = 18.7 \pm 0.4 \mu M$). Error bars represent $\pm$ SD ($n = 3$).

Table 6. Cytotoxic activity of CAOD against A2780 ovarian cancer cells (MTT, 48 h). Values are mean $\pm$ SD of three independent experiments.

Compound	Conc. (μM)	Cell Viability (%)	Inhibition (%)	IC_{50} (μM)	Ref. IC_{50}
Control	0	100	0	—	—
CAOD	6.25	82	18	18.7 ± 0.4	Cisplatin: 12.4
CAOD	12.5	64	36		
CAOD	25	41	59		
CAOD	50	22	78		
CAOD	100	9	91		

the scan ($R_p = 0.107$, $R_{wp} = 0.136$, $\chi^2 = 1.37$). The pattern has two components. A broad, intense feature at $2\theta = 26.75^\circ$ ($d = 3.33 \text{ \AA}$) dominates the trace, and weaker diffuse features sit at 11.69° ($d = 7.56 \text{ \AA}$) and 24.03° ($d = 3.70 \text{ \AA}$). Widths of 1.4–

3.6° 2θ mark these three as scattering from poorly ordered material. Eight sharp reflections appear at 29.57 , 31.78 , 31.99 , 45.62 , 56.66 , 66.42 , 75.44 and 84.12° 2θ , with widths of only 0.13 – 0.42° . The solid is therefore semi-crystalline: nanocrystalline

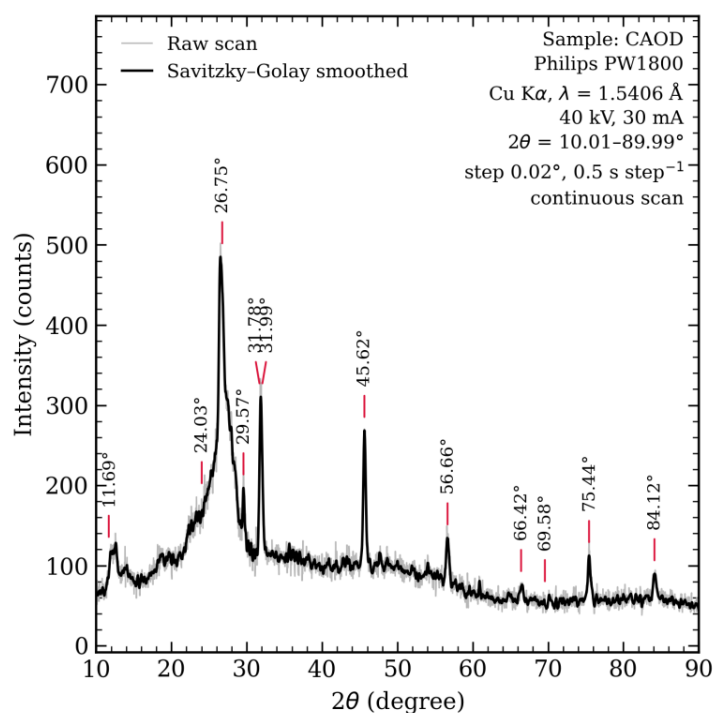


Fig. 8. Powder X-ray diffractogram of CAOD (Philips PW1800, Cu $K\alpha$, 40 kV, 30 mA; $2\theta = 10.01$ – 89.99° , step 0.02° , 0.5 s per step). The grey trace is the recorded scan, the black trace is Savitzky–Golay smoothed, and the marks give the fitted $K\alpha_1$ peak positions listed in Table 7.

Table 7. Reflections fitted in the powder pattern of CAOD ($K\alpha_1$ components of the profile refinement; $R_p = 0.107$, $R_{wp} = 0.136$, $\chi^2 = 1.37$) and apparent Scherrer crystallite sizes ($K = 0.9$, $\lambda = 1.5406 \text{ \AA}$, no correction for instrumental broadening).

Pos. (2θ)	d ($\text{\AA}$)	FWHM (2θ)	Rel. Int. (%)	D (nm)	Assignment
11.69	7.563	3.6	24.6	— ^a	diffuse
24.03	3.701	1.8	17.7	— ^a	diffuse
26.75	3.330	1.39	100.0	— ^a	diffuse; π – π stacking
29.57	3.019	0.13	26.0	63.2	—
31.78	2.814	0.15	59.7	55.1	NaCl (200)
31.99	2.796	0.16	55.7	51.7	—
45.62	1.987	0.28	60.6	30.8	NaCl (220)
56.66	1.623	0.42	16.1	21.5	NaCl (222)
66.42	1.406	0.40	4.1	23.7	NaCl (400)
69.58	1.350	— ^b	2.1	—	—
75.44	1.259	0.32	20.0	31.4	NaCl (420)
84.12	1.150	0.40	12.9	26.8	NaCl (422)

^a Diffuse feature, excluded from the Scherrer average. ^b FWHM not resolved for this weak reflection. Mean D over the eight sharp reflections = 38.0 nm.

domains are dispersed in a largely disordered matrix. The spacing of the dominant feature, 3.33 Å, lies in the range typical of π - π stacking between aromatic planes, so the disordered fraction keeps face-to-face contact between the azo-linked aromatic units.

Six of the sharp reflections match the strongest lines of sodium chloride (JCPDS 05-0628). The observed spacings of 2.814, 1.987, 1.623, 1.406, 1.259 and 1.150 Å compare with 2.821, 1.994, 1.628, 1.410, 1.261 and 1.151 Å for the 200, 220, 222, 400, 420 and 422 planes of halite. Residual NaCl is expected in this chemistry. It forms stoichiometrically during diazotisation and azo coupling, it precipitates together with the dye, and it is almost insoluble in the ethanol used for recrystallisation. The two remaining sharp lines, at 29.57 and 31.99°, do not belong to the halite pattern and are left unassigned.

The apparent crystallite size D was estimated with the Scherrer equation, $D = K\lambda/(\beta \cos \theta)$, using $K = 0.9$ and $\lambda = 1.5406$ Å [45]. Only the eight sharp reflections were used; the diffuse features and the unresolved line at 69.58° were excluded. D ranges from 21.5 to 63.2 nm and averages 38 nm (Table 7). No correction was made for instrumental broadening, so these values are lower-bound estimates. For the halite-matched lines, D measures the size of the residual salt crystallites.

Fig. 9 shows the surface of the same solid by FESEM. At 10,000× the sample consists of irregular plate-like aggregates several micrometres across, stacked and fused at their edges (Fig. 9a). At

50,000× the plate surfaces carry near-spherical nanogranules (Fig. 9b). Spot measurements made with the microscope software gave granule diameters of 20.10, 24.56, 26.80 and 37.96 nm, and one isolated rounded particle of 93.81 nm. Automated analysis of the same micrograph (thresholding with watershed separation, calibrated on the embedded 200 nm scale bar) resolved 107 granules with diameters of 10–54 nm, a mean of 16.5 ± 6.3 nm and a median of 14.5 nm. The size distribution and its Gaussian fit are inset in Fig. 9b.

The two probes agree. The plates are the organic solid, largely disordered but holding the π -stacked contact seen at $d = 3.33$ Å. The nanogranules on the plate surfaces (10–54 nm) match the sharp-line crystallite sizes from the Scherrer analysis (21–63 nm), so at least part of the surface decoration is assigned to the residual salt dispersed over the organic plates. Elemental mapping would make this assignment definitive.

CONCLUSION

An azo-bridged 1,3-oxazepine-4,7-dione has been prepared in four steps and in 86 % yield by cyclising an azo Schiff base with maleic anhydride. The ring closure is established by three independent observations: the carbonyl band at 1682 cm^{-1} that the precursor lacks, the disappearance of the azomethine singlet at 8.79 ppm together with a new one-proton singlet at 5.56 ppm, and the sp^3 carbon at 66.81 ppm alongside the two carbonyl carbons at 168.36 and

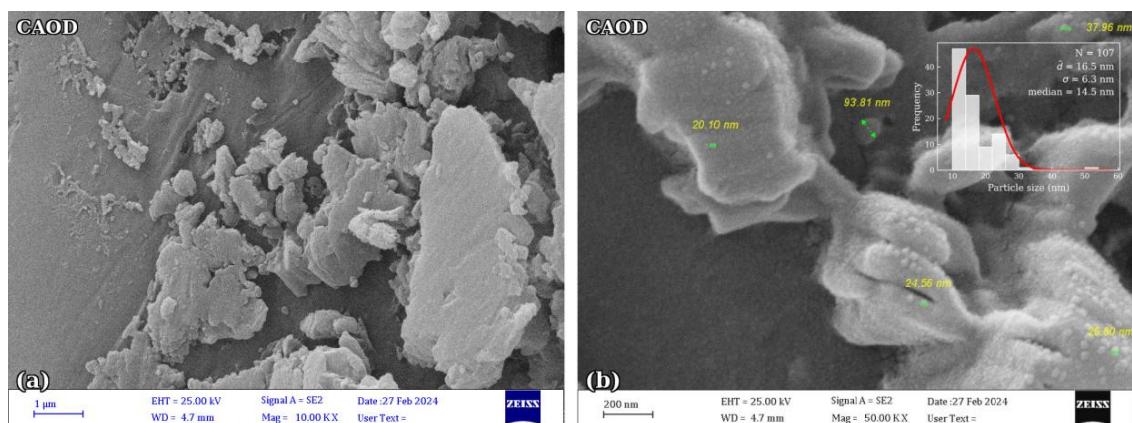


Fig. 9. FESEM micrographs of CAOD (ZEISS, 25.00 kV, SE2 detector, working distance 4.7 mm): (a) 10,000×, scale bar 1 μm; (b) 50,000×, scale bar 200 nm. Green markers are the microscope spot measurements (20.10–93.81 nm). Inset: granule-size distribution from automated segmentation of (b), with a Gaussian fit ($N = 107$, mean 16.5 ± 6.3 nm, median 14.5 nm).

163.75 ppm. The calculations answer the question that motivated the work. Inserting a *trans* azo bridge into this scaffold narrows the frontier gap from 4.079 to 3.083 eV, converts a HOMO that was 93 % localised on one ring into one spread across four fragments, and leaves a softer, more electrophilic and more polar molecule. Biologically the series peaks at the Schiff base rather than at the target: CBHB gives 16 and 20 mm zones, azo coupling almost abolishes them, and cyclisation restores about two-thirds of the Gram-positive response. Completing the cytotoxicity dataset, confirming the formula by mass spectrometry and establishing the azo regiochemistry are the immediate next steps.

CAOD exhibited concentration-dependent cytotoxicity against A2780 ovarian cancer cells with an IC_{50} of $18.7 \pm 0.4 \mu M$, approximately 1.5-fold higher than cisplatin ($12.4 \mu M$). This activity is consistent with the narrow HOMO–LUMO gap and the high electrophilicity predicted by DFT.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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