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$FeCl_3$ catalyzed tandem synthesis of optically pure sugar based benzimidazoles

Sarita Mishra[a], Soumik Roy[a†], Rina Ghosh*[a], Diganta Dey[b,c] and Banasri Hazra[b$]

[a]Department of Chemistry, Jadavpur University, Kolkata-700 032, India

E-mail: ghoshrina@yahoo.com, rina.ghosh@jadavpuruniversity.in

[b]Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India

[c]Department of Microbiology, Ashok Laboratory Clinical Testing Centre Private Limited, Kolkata-700 068, India

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A chemo-selective synthesis of optically pure sugar based benzimidazole from reaction of glycal-2-carboxaldehyde and *ortho*-phenylene diamine by $FeCl_3$ catalyzed tandem imination, cyclisation followed by green oxidation by aerial O_2 is reported. Some selected tailor-made chiral benzimidazoles were screened for antimicrobial activity against clinically isolated drug-resistant bacteria, as well as fungi obtained from superficial dermal infections.

Keywords: $FeCl_3$, catalyzed, glycal, benzimidazole, tandem.

Introduction

The importance of nitrogen-containing heterocycles originates from their wide occurrence in Nature and broad application in chemistry, biology, and material sciences[1]. The most prominent benzimidazole (BZM) compound in Nature is *N*-ribosyl 5,6-dimethylbenzimidazole which serves as an axial ligand for cobalt in Vitamin B12[2a] and has generated considerable interest in the area of BZM based nucleoside and nucleotides or the corresponding analogs[2b-e].

BZM and its derivatives are widespread in many therapeutic areas and have versatile pharmacological properties[3] such as antiviral[3a], (Hep.C, HIV), antibacterial[3b], antifungal[3c], antitumor[3d], antihelminthics[3e], etc. BZMs have importance as analgesic[3f] or cardiovascular agents[3g] and are useful in neuroresearch[3h], endocrinology[3i] and also in opthamology[3j]. Apart from these the antiallergic[3k] and antihistaminic[3l] activity of compounds containing BZM moiety have also been reported. BZM frameworks are also finding many applications in several other fields[4].

The BZM motif occurs in many approved and investigational drugs (Fig. 1)[5]. Some established drugs like omeprazole, pantoprazole, lansoprazole, dexlansoprazole (dexillant-enantiomer of lansoprazole, Fig. 1) and esomeprazole (nexium, Fig. 1) are also having common benzimidazole sulfoxide framework and are used as proton pump inhibitors (PPIs)[6] in mammals, especially in human beings.

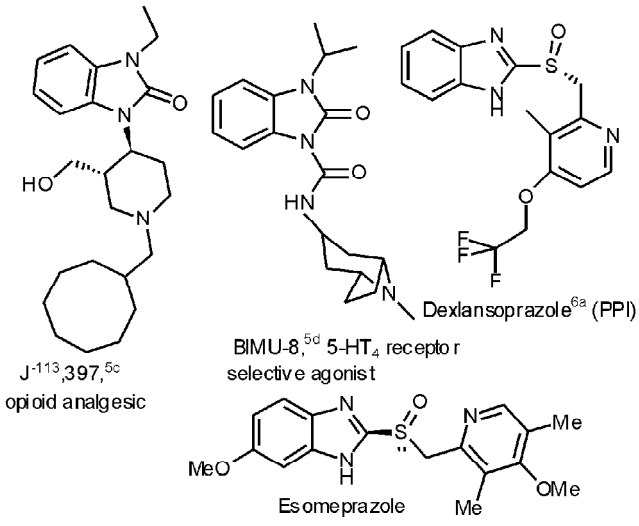


Fig. 1. Drugs containing chiral benzimidazole frameworks.

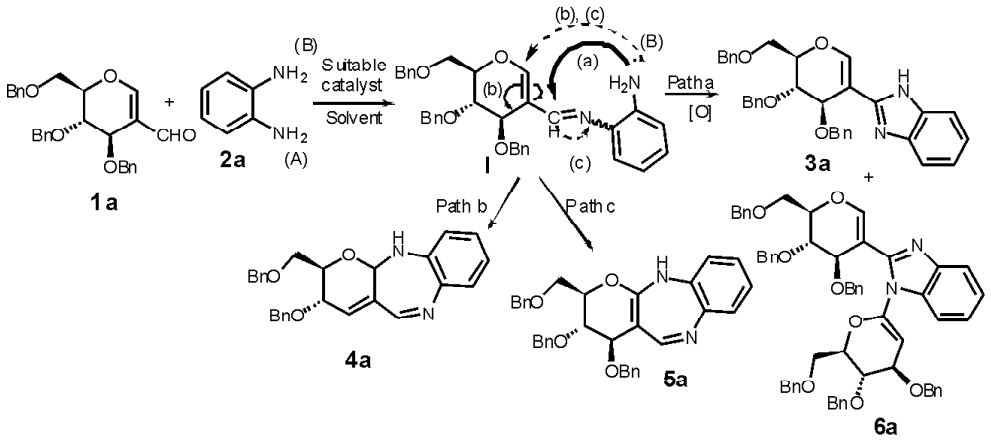
†Current affiliation: Department of Chemistry, Amity University, Kolkata-700 135, India.
$Retired Professor.

Many synthetic nucleosides[2b-e,3f,7] and nucleotides[2b-e] of BZM are known. Structurally diverse molecules having novel skeletons are of primal requirement for drug discovery. Development of improved synthetic methodologies, particularly tandem ones[8] involving multiple chemical transformations in one-pot by a single catalyst, is of immense importance for generation of library compounds. The transition metal catalyzed cycloisomerisation approach is a powerful tool for the assembly of diverse heterocyclic frameworks[9]. Over the past few years, iron salts[10] have emerged as promising catalysts and thus have attracted increasing research interest of chemists. Due to the low price, non-toxicity, ready availability and environmentally friendly character recently, particularly $FeCl_3$ has received much attention in the synthesis of heterocyclic compounds[10a,c,11]. In connection to our work on synthesis of heterocycles[12] and also on glycal systems[13], we report herein chemo-selective synthesis of chiral benzimidazoles by cyclocondensation of OPDs (*o*-pheylene diamines) with tri-*O*-benzyl/methyl glycal-2-carboxaldehydes by $FeCl_3$ catalyzed tandem imination, cyclisation and aerial oxidation in refluxing CH_3CN in one-pot (Table 1, Schemes 1 and 2) along with the results of antimicrobial assays of some selected chiral benzimidazoles (Tables 2-4 and Fig. 4-7).

Results and discussion

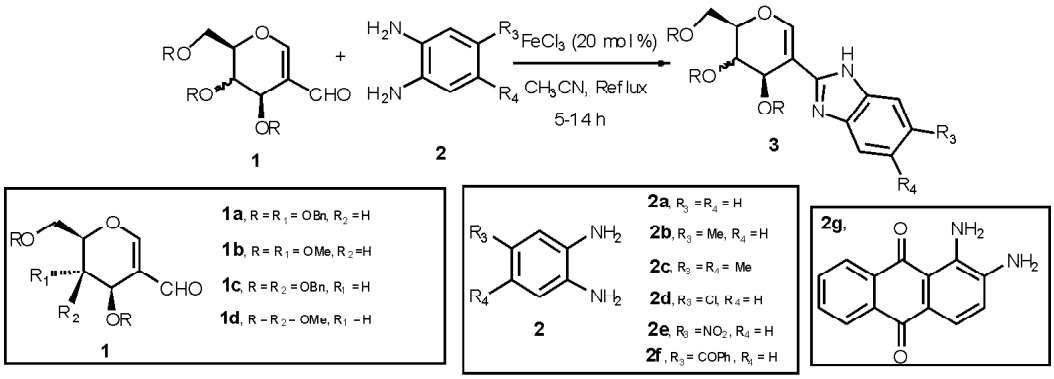
SiO_2-$FeCl_3$/H_2O_2[14a], sulfonic acid functionalized imidazolium salts-$FeCl_3$[14b], I_2 alone or in combination with other reagents[14c,d] and $ZrOCl_2.8H_2O$[14e] are known to form benzimidazoles from reaction of aldehydes and *o*-phenylenediamines. Of these I_2[15a], and $FeCl_3$[15b], and other catalyst like $InCl_3$[15c], are reported to give *O*- or -*C* Ferrier rearrangements in glycals. Benzimidazole as well as benzdiazepine is generated by $FeCl_3$ catalyzed oxidative condensation of α,β-unsaturated aldehyde (cinnamaldehyde) and *o*-phenylene diamine (our unpublished observation). $InCl_3$ is also known to catalyze the synthesis of sugar derived 1,5-benzodiazepine from reaction of sugar derived α,β-unsaturated-δ-hydroxyaldehyde (non-glycal system) and *o*-phenylenediamine[15d].

Initially, we were thus in search of a suitable reagent or reagent system that would not cause direct Ferrier type rearrangement in the 3,4,6-tri-*O*-benzyl-D-glucal-2-carboxaldehyde (**1a**) by *o*-phenylenediamine (**2a**) but could generate preferably either benzimidazole or benzodiazipines through reaction of the initially formed imine intermediate **I** by attack of the other free amino group on the imino carbon [pathway (a)] or on the anomeric carbon [pathway (b) or (c)], respectively, followed by oxidation (Scheme 1). With this end in view, 3,4,6-tri-*O*-benzyl-glucal-2-carboxaldehyde (**1a**) was reacted separately with (**2a**) in the presence of several catalysts like $InCl_3$, $La(OTf)_3$, $ZrOCl_2.8H_2O$, I_2 and $FeCl_3$ under different reaction conditions. The best yield of the corresponding optically pure benzimidazole (**3a**, 90%) was obtained by refluxing a mixture of **1a** and **2a** in CH_3CN in the presence of 20 mol% $FeCl_3$(Table 1). Neither the corresponding benzodiazepines **4a** or **5a** nor any dialkylated benzimidazole **6a** could be traced out in the crude product. Thus, here the condi-



Scheme 1. Probable anticipated pathways.

Table 1. $FeCl_3$ catalyzed one-pot synthesis of library of glycal fused chiral benzimidazoles
[a]See Fig. 2 for the structure of products



Entry	Glycal aldehyde (**1**)	OPD (**2**)	Product (**3**)	Time (h)	Yield (%)	Nature/m.p. (°C)
1	**1a**	**2a**	**3a**[7f]	6	90	Solid/110
2	**1a**	**2b**	**3b**[7f]	8	81	Foam
3	**1a**	**2c**	**3c**[7f]	8	78	Foam
4	**1a**	**2d**	**3d**	6.5	85	Foam
5	**1a**	**2e**	**3e**	7	81	Foam
6	**1a**	**2f**	**3f**	10	77	Foam
7	**1a**	**2g**	**3g**[7f]	11	76	Solid/126
8	**1b**	**2a**	**3h**[7f]	6	82	Syrup
9	**1b**	**2c**	**3i**	7	70	Syrup
10	**1b**	**2d**	**3j**	6	81	Syrup
11	**1b**	**2e**	**3k**	5	70	Syrup
12	**1b**	**2g**	**3l**[7f]	12	71	Solid/289–291
13	**1c**	**2a**	**3m**[7f]	7	80	Syrup
14	**1c**	**2c**	**3n**	7	48	Syrup
15	**1c**	**2e**	**3o**	7	78	Syrup
16	**1c**	**2f**	**3p**[7f]	11	78	Foam
17	**1c**	**2g**	**3q**[7f]	14	71	Solid/166
18	**1d**	**2g**	**3r**[7f]	10	80	Solid/236

[a]All reactions were performed by using 1.0 equiv. of aldehyde, 1.5 equiv. of *ortho*-phenylenediamine and 20% of $FeCl_3$ in CH_3CN under reflux condition; the yields reported in the table are the isolated yields of the compounds.

tion is highly chemoselective. It is worthy to mention here that Maiti *et al.*[7f] have also reported recently the synthesis of benzimidazoles (including chiral ones) from reactions of aldehydes including glycal derived aldehydes and *o*-phenylenediamines based on $VO(acac)_2$-$CeCl_3$-$Ti(OBu)_4$ Combo catalyst. We have proved unequivocally the structure of the sugar-based benzimidazole **3a** by XRD analysis[16] (CCDC No. 905529) (Fig. 3a) and NMR (^1H and ^{13}C, 1D and 2D) analysis. This result incited us to further explore this methodology towards synthesis of diversified optically pure benzimidazole scaffolds.

Under the standard reaction condition, various glycal-2-carboxaldehydes **1**(**a**-**d**) in reaction with *o*-phenylenediamine **2a** or its analogs **2**(**b**-**g**) generated the corresponding optically pure benzimidazoles (**3**, Table 1) in good to excellent yields (70–90%). Corresponding benzodiazepines or bis-sugar derivatives were not formed in any of these cases. 3,4,6-Tri-*O*-benzyl-D-glucal-2-carboxaldehyde (**1a**) in reac-

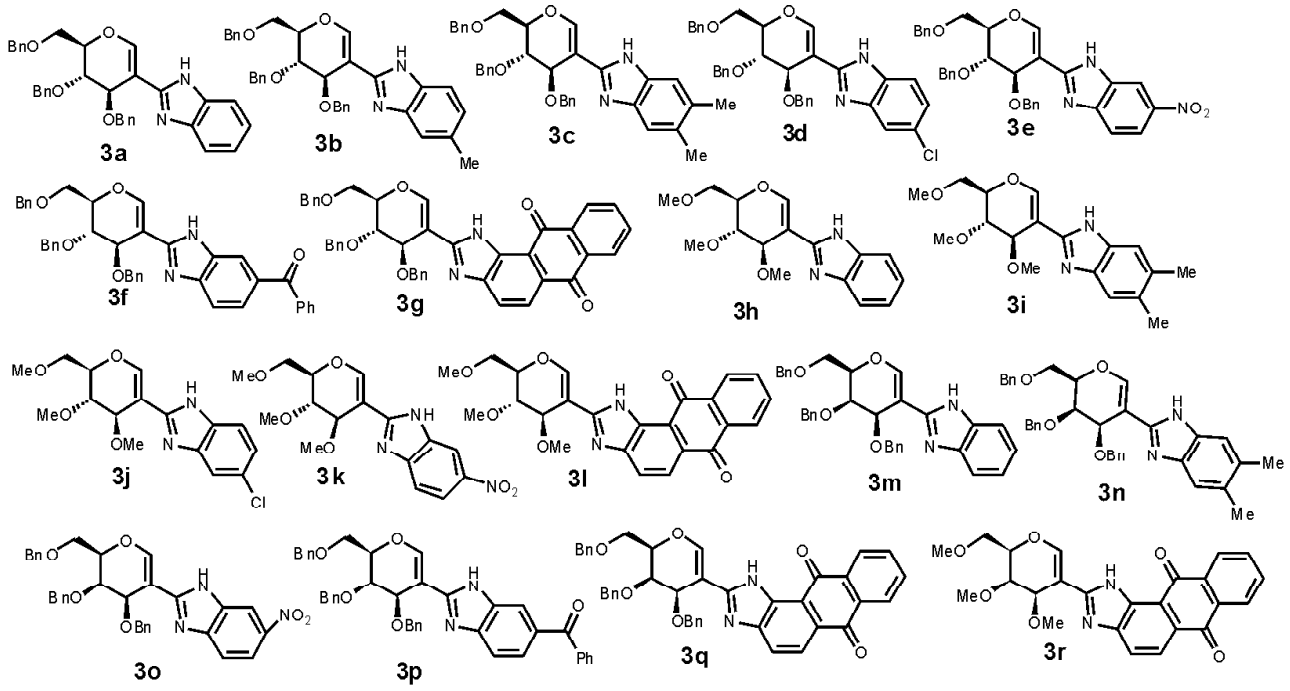


Fig. 2 Structure of glycal fused chiral benzimidazole products.

tion with *o*-phenylenediamines containing substituents having +I effect **2b** and **2c** or having –I and +R effects **2d** or –I, –R effects **2e** and **2f** afforded the corresponding benzimidazoles **3b-3f** in high yields (77–85%). Similarly, 3,4,6-tri-*O*-methyl-glucal-2-carboxaldehyde **1b** also reacted efficiently under the optimized condition with **2a** and **2c**-**2e** furnishing the corresponding optically pure benzimidazoles (**3h** and **3i**-**3k**) in good yields (70–82%). The efficacy of the present procedure was further proved from the reaction of *O*-benzylated galactal-2-carboxaldehyde **1c** with **2a**, **2c** or its electron deficient analogs (**2e** and **2f**), which uniformly generated the corresponding optically pure benzimidazoles **3m**, **3o** and 3**p** respectively in high yields (78–80%) [except **3n**, that was obtained in moderate yield]. The applicability of the present method was further explored for synthesis of sugar based benzimidazoles from reactions of aldehydes (**1a**-**1d**) with 1,2-diamino-9,10-anthraquinone **2g** which gave the corresponding desired optically pure products, **3g**, **3l**, **3q** and **3r**, respectively in quite high yields (71–80%). In general the rate of reactions of 4-benzoyl-*o*-phenylenediamine **2f** or the *o*-diaminoquinone **2g** were slower (reaction time: 10–14 h) incomparison with those of the other *o*-diaminoaromatic compounds (5–8 h). It is also to be noted that all the reactions under Table 1 were clean and high yielding compared to those

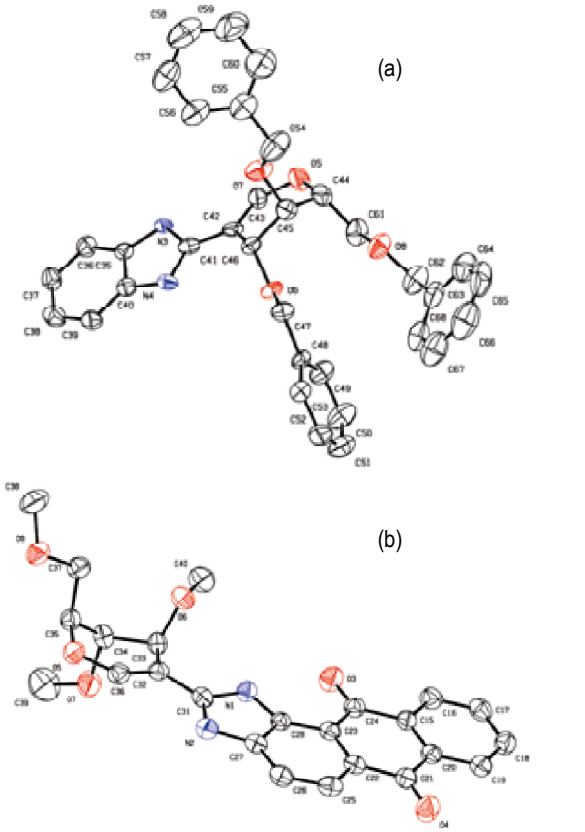


Fig. 3. (a) Crystal structure (ORTEP diagram) of compound **3a**, (b) crystal structure of compound **3l**.

reported earlier[7f]. We also want to mention that the structure and the stability of the specific tautomeric form **3l** has been confirmed unequivocally by XRD analysis[16], CCDC No. 905528 (Fig. 3b) of its single crystal.

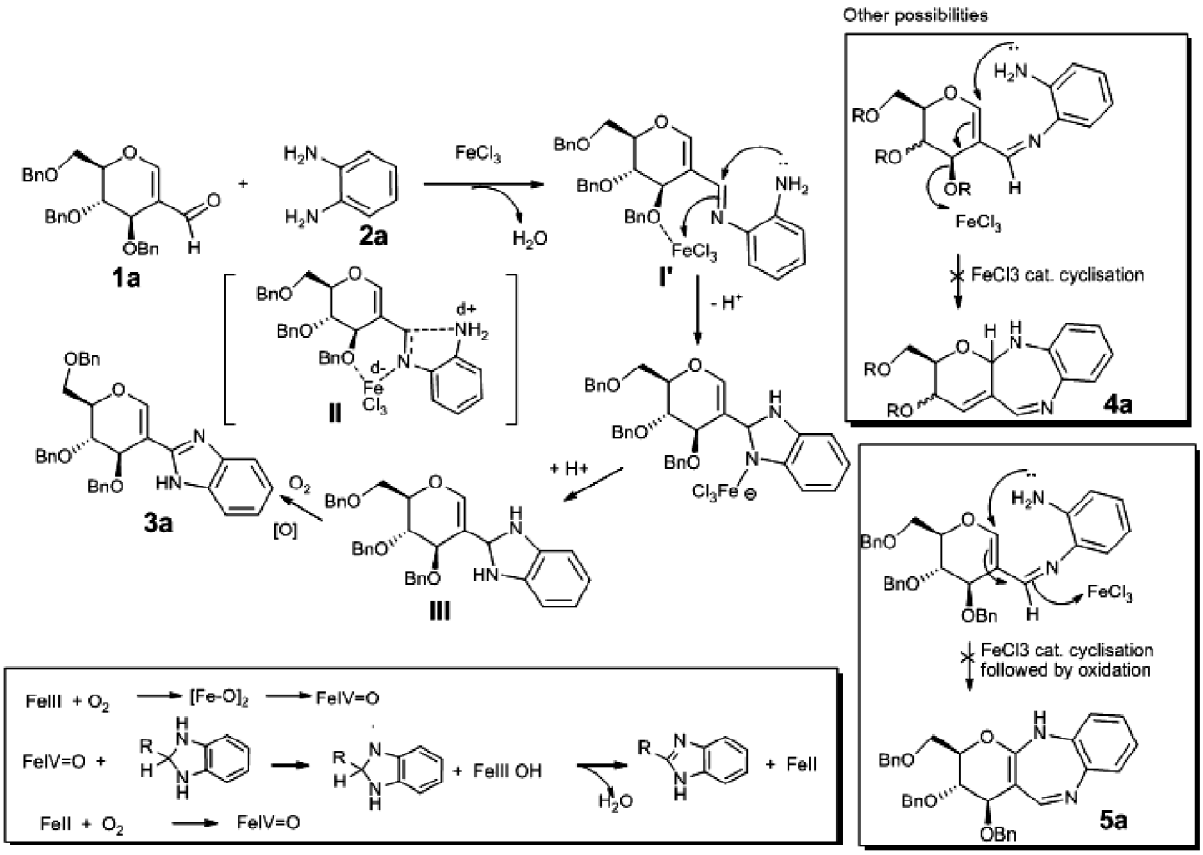
It is interesting to note that *o*-phenylenediamine **2b** or **2d** containing Me or Cl group at C-4 in reaction with sugar aldehydes generated in all cases the corresponding 5-methyl- or 5-chloro-benzimidazoles (Table 1). On the other hand, *o*-phenylenediamine analogues containing electron withdrawing groups (–I, –R) such as -NO_2 or -COPh at C-4 (**2e** or **2f**) reacted with the glycal-2-carboxaldehydes affording the corresponding 6-NO_2- or 6-benzoylbenzimidazoles.

The plausible mechanistic pathway for the formation of compounds (**3**) is depicted in Scheme 2. The reaction is most probably initiated by $FeCl_3$ catalyzed formation of the intermediate Schiff's base **I**, which in its *s-trans* form can proceed via formation of a 6-membered chelate through coordination of Fe(III) with imine N and C_3-O of the glycal moiety, eventually producing the corresponding dihydrobenzimidazole intermediate **II**. **II** is finally oxidized *in situ* by Fe(III) catalyzed oxidation where aerial O_2 plays the key role as shown in the inset of Scheme 2.

The most important point to be noted is that just refluxing of the reaction mixture using a guard tube can produce the benzimidazole products (**3**) in high yields. Continuous flow (or bubbling) of O_2 is not necessary for the oxidation step (as this does not increase the yield of products).

That O_2 plays the key role and the oxidation is mediated by Fe(III) are also evidenced from the fact that the reaction fails to give any product in the absence of $FeCl_3$, and also that a small amount of the desired product is obtained when a reaction was performed under N_2-atomosphere.

As has been mentioned earlier benzimidazoles are considered as a promising class of bioactive heterocyclic compounds exhibiting a wide range of biological activities[3]. Benzimidazole urea, discovered by the use of structure-guided



Scheme 2. Proposed mechanism for the synthesis of **3a.**

Table 2. Antimicrobial activity of the synthesized benzimidazole derivatives on microorganisms isolated from skin and subcutaneous infection

Microorganism; Source	Inhibition zones of microbial growth (mm)[a]										MIC values (μg/ml)						
	Benzimidazole derivatives (0.2 mg/well)							Standard drugs (0.02 mg/well)			Benzimidazole derivatives						
Gram-positive bacteria:	**3a**	**3c**	**3o**	**3n**	**3l**	**3g**	**3r**	C	G	F	**3a**	**3c**	**3o**	**3n**	**3l**	**3g**	**3r**
Staphylococcus aureus (MSSA);	B	7	9	b	9	8	10	16	16	ND	>512	256	128	>512	128	256	128
Vaginal swab																	
Staphylococcus aureus (MRSA);	6	b	7	b	B	8	B	22	16	ND	>512	>512	256	>512	>512	256	>512
Wound swab:																	
Streptococcus pyogenes;	8	8	B	6	7	10	8	24	22	ND	256	256	>512	256	256	128	256
Pus																	
Enterococcus faecalis;	B	b	7	b	B	6	8	10	19	ND	>512	>512	256	>512	>512	256	256
Pus																	
Gram-negative bacteria																	
Escherichia coli (ESBL producer);	B	6	6	b	B	7	8	9	16	ND	>512	256	>512	>512	>512	256	256
Vaginal swab:																	
Klebsiella pneumoniae (ESBL producer);	B	6	b	b	B	10	7	10	16	ND	>512	256	>512	>512	>512	128	256
Wound swab																	
Proteus mirabilis (ESBL non-producer);	7	b	8	6	8	9	B	26	22	ND	256	>512	256	256	128	256	>512
Wound swab																	
Pseudomonas aeruginosa;	B	b	8	7	9	6	B	22	18	ND	>512	>512	256	256	128	256	>512
Pus																	
Fungal strains:																	
Candida albicans;	7	8	6	9	8	6	10	ND	ND	18	256	256	>512	256	256	>512	128
Skin scraping																	
Candida tropicalis;	10	11	B	15	11	9	12	ND	ND	14	128	128	>512	128	128	128	128
Pus																	

[a]Including diameter of well (5 mm). "b": No activity. ND: Not Determined. "C": Ciprofloxacin. "G": Gentamicin. "F": Fluconazole, MSSA: methicillin sensitive *Staphylococcus aureus*, MRSA: methicillin resistance *S. aureus*, ESBL: extended spectrum β-lactamase.

design and structure-activity relationships, was found to be dual targeting inhibitors of DNA gyrase and topoisomerase IV, with potent antibacterial activity against a wide spectrum of relevant pathogens responsible for hospital- and community-acquired infections[17]. In the present study, seven benzimidazole derivatives were selected for prospective antimicrobial assessment. Bacterial and fungal isolates were taken from patients suffering with superficial and subcutaneous infections, constituting four Gram-positive, four Gram-negative and a two *Candida* spp. (Table 2). The collected bacterial strains, when tested for their antibiotic resistance profile, were found to be resistant against multiple antibiotics (Table 3). One of the bacteria was methicillin resistant *Staphylococcus aureus* (MRSA) and two of the Gram-negative bacilli were extended spectrum β-lactamase (ESBL) producers[18], which are currently posing global problem in clinical therapeutics due to their multi-drug resistant characteristics.

In the present study, the compound **3g** responded, more or less, to all the tested organisms, while the other six compounds exhibited 'mild' to 'no activity' against the bacterial strains (Table 2, Fig. 4). However, all the compounds responded positively to both the *Candida* spp. (Table 2, Fig. 5). The MIC determined for the compounds showed the lowest value at 128 μg/ml (Table 2). The tested dose of 200 μg/well, although 10-fold higher than the standard drugs, would be compatible for dermal medication regimens, particularly for treatment of fungal diseases, since the test organisms were isolated from superficial and subcutaneous infections. Hence, the selected benzimidazoles might be developed for prospective therapeutic application against candidiasis.

Invasive fungal infections have become major causes of morbidity and mortality among immune-suppressed patients. *Candida* species are now a frequent cause of nosocomial blood stream infections in critically ill patients even in developed countries[19]. Therefore, we decided to further investigate the extent of DNA damage in *Candida tropicalis* following treatment with two of the derivatives (**3n** and **3r**) by using alkaline comet assay. The study was done in terms of migrated DNA fragments by using fifteen commonly used parameters, taking hydrogen peroxide as a standard control. Interestingly, we detected increased levels of DNA fragmentation in fungal cells treated with benzimidazoles as compared with the untreated (negative control) cells ($p < 0.05$)

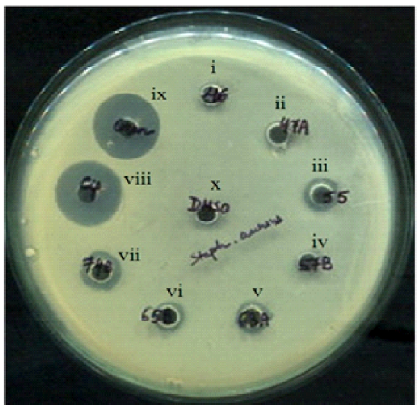


Fig. 4. Antibacterial activity of benzimidazole derivatives viz. (i) **3a**, (ii) **3c**, (iii) **3o**, (iv) **3n**, (v) **3l**, (vi) **3g** and (vii) **3r** against *Staphylococcus aureus* (MSSA), (viii) Ciprofloxacin and (ix) gentamicin, at 0.02 mg/well, were used as positive controls and 50 μL DMSO was used as (x) vehicle control.

Table3. *In vitro* antibiotic resistance pattern of the clinically isolated bacteria

Bacteria	Specimen	Antibiotic resistance
Staphylococcus aureus (MSSA)	Vaginal swab	E, Nx, Ca, Cfm
Staphylococcus aureus (MRSA)	Wound swab	Cfx, Nx, Pt, Ca, cfm, Cf
Streptococcus pyogenes	Pus	Ca, Cfm, V, Cl
Enterococcus faecalis	Pus	As, Amc, Cp, Cfx, Cip, Lv, Nx, Of, Sfx
Escherichia coli (ESBL producer)	Vaginal swab	Cfx, Cip, Nx, Sfx, Ca
Klebsiella pneumoniae (ESBL producer)	Wound swab	As, Amc, Cfx, Cip, Cl, Nx, Of, Pt, Sfx, Ca, Cfm, Cfs, Ao
Proteus mirabilis (ESBL non-producer)	Wound swab	Do, Ca
Pseudomonas aeruginosa	Pus	As, Amc, Cl, Do, Cfm, Ao

MSSA: methicillin sensitive *Staphylococcus aureus*, MRSA: methicillin resistance *S. aureus*, ESBL: extended spectrum β-lactamase, As: Ampicillin/Sulbactum (10/10 μg), Amc: Amoxy-Clavulanic acid (20/10 μg), Ao: Aztreonam (30 μg), Ca: Ceftazidime (30 μg), Cfm: Cefixime (5 μg), Cfs: Cefoperazone/Sulbactum (75/30 μg), Cf: Cefoxitin (30 μg), Cp: Cephalexin (30 μg), Cfx: Cefuroxime (30 μg), Cip: Ciprofloxacin (5 μg), Cl: Ceftriaxone (30 μg), Do: Doxycycline (30 μg), E: Erythromycin (30 μg), Lv: Levofloxacin (5 μg), Nx: Norfloxacin (10 μg), Of: Ofloxacin (5 μg), Pt: Piperacillin/Tazobactum (100/10 μg), Sfx: Sparfloxacin (5 μg), V: Vancomycin (30 μg).

Table 4. Evaluation of DNA damaging effect of the synthesized benzimidazole derivatives on *Candida tropicalis* by Comet assay

	Negative control Mean±SE[a]	3n Treated Mean±SE (p-value[b])	3r Treated Mean±SE (p-value)	Positive control Mean±SE (p-value)
Comet length (μm)	2.72±0.07	4.50±0.15 (1.59×10^{-11})	4.69±0.22 (4.04×10^{-9})	6.85±0.36 (8.57×10^{-11})
Comet height (μm)	2.93±0.06	3.76±0.11 (8.63×10^{-8})	3.60±0.09 (7.04×10^{-7})	3.71±0.14 (1.22×10^{-5})
Comet area (μm²)	7.80±0.28	15.49±0.72 (1.38×10^{-10})	15.73±1.06 (1.82×10^{-7})	22.62±1.95 (1.65×10^{-7})
Comet intensity	10257.23±267.42	14044.18±1138.03 (3.58×10^{-3})	14253.32±1590.77 (2.13×10^{-2})	20265.41±1962.47 (4.77×10^{-5})
Comet mean intensity	76.45±3.13	50.95±3.29 (1.43×10^{-6})	48.97±2.81 (7.19×10^{-8})	49.80±2.24 (3.18×10^{-8})
Head diameter (μm)	2.61±0.09	3.20±0.15 (1.34×10^{-3})	2.79±0.19 (0.39)	3.42±0.29 (1.24×10^{-2})
Head area (μm²)	7.71±0.27	13.23±0.76 (3.04×10^{-7})	14.09±1.03 (3.83×10^{-6})	14.19±1.60 (6.01×10^{-4})
Head mean intensity	72.96±3.17	41.45±2.27 (8.95×10^{-10})	35.56±2.75 (3.74×10^{-11})	41.31±3.32 (2.11×10^{-8})
%DNA in head	94.65±1.84	70.38±3.11 (1.03×10^{-7})	64.52±3.91 (9.68×10^{-8})	52.39±5.28 (5.040×10^{-8})
Tail length (μm)	0.21±0.06	1.30±0.13 (2.69×10^{-8})	1.89±0.22 (8.98×10^{-8})	3.43±0.30 (3.12×10^{-10})
Tail area (μm²)	0.09±0.02	2.26±0.33 (1.45×10^{-6})	1.65±0.29 (2.05×10^{-5})	8.43±1.39 (6.09×10^{-6})
Tail intensity	573.45±197.95	4295.41±637.95 (8.54×10^{-6})	4834.45±810.80 (3.38×10^{-5})	9549.27±1252.65 (4.18×10^{-7})
% DNA in tail	5.35±1.84	29.62±3.11 (1.03×410^{-7})	35.48±3.91 (9.68×10^{-8})	47.61±5.28 (5.04×10^{-8})
Tail moment	0.06±0.03	1.70±0.27 (3.77×10^{-6})	3.16±0.59 (3.13×10^{-5})	7.70±1.13 (1.05×10^{-6})
Olive moment	0.17±0.06	1.55±0.17 (5.07×10^{-8})	2.04±0.25 (2.00×10^{-7})	5.12±0.65 (1.52×10^{-7})

[a]SE: Standard error. [b]p-value is the result of comparison with negative control by independent, two tailed, Studentt-test. $p < 0.05$ is considered as statistically significant.

(Table 4 and Fig. 6). Among the fifteen calculated comet assay parameters, tail length, tail intensity, % DNA in tail, and tail moment were depicted in box-whisker plots, for the visual comparison between untreated control, **3n**-treated, **3r**-treated, and hydrogen peroxide treated fungal cells (Fig. 7). DNA damage may result in apoptosis or induction of mutations which are believed to be the mode of antifungal activity of this class of heterocyclic compounds.

In fact, there are very few reports on comet assay performed for the assessment of drug-induced DNA fragmentation in *Candida* spp.[20], and to the best of our knowledge, the present study is one of the first such works published on benzimidazoles.

Conclusion

In summary, we have demonstrated an efficient chemoselective clean tandem synthesis of sugar based optically pure benzimidazoles from reactions of glycal-2-

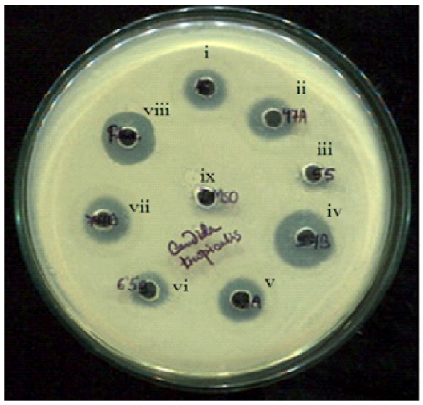


Fig. 5. Antifungal activity of benzimidazole derivatives viz. (i) **3a**, (ii) **3c**, (iii) **3o**, (iv) **3n**, (v) **3l**, (vi) **3g** and (vii) **3r** against *Candida tropicalis*. (viii) Fluconazole (0.02 mg/well) was used as positive control and 50 µL DMSO was used as (ix) vehicle control.

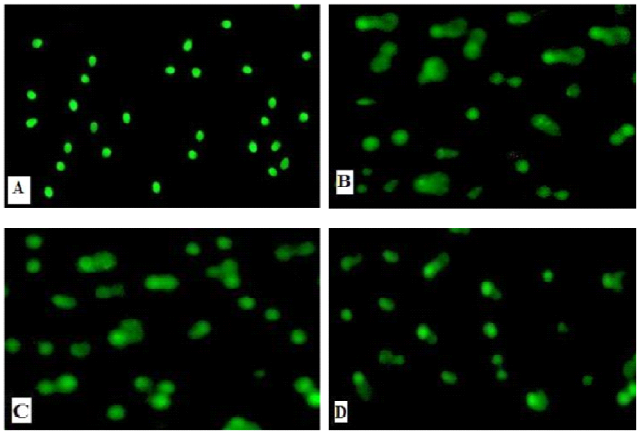


Fig. 6. Extent of DNA damage in *Candida tropicalis*. (A) Untreated fungal cells; (B) 100 µL hydrogen peroxide treated fungal cells; (C) **3n** (200 µg/ml) treated fungal cells; (D) **3r** (200 µg/ml) treated fungal cells.

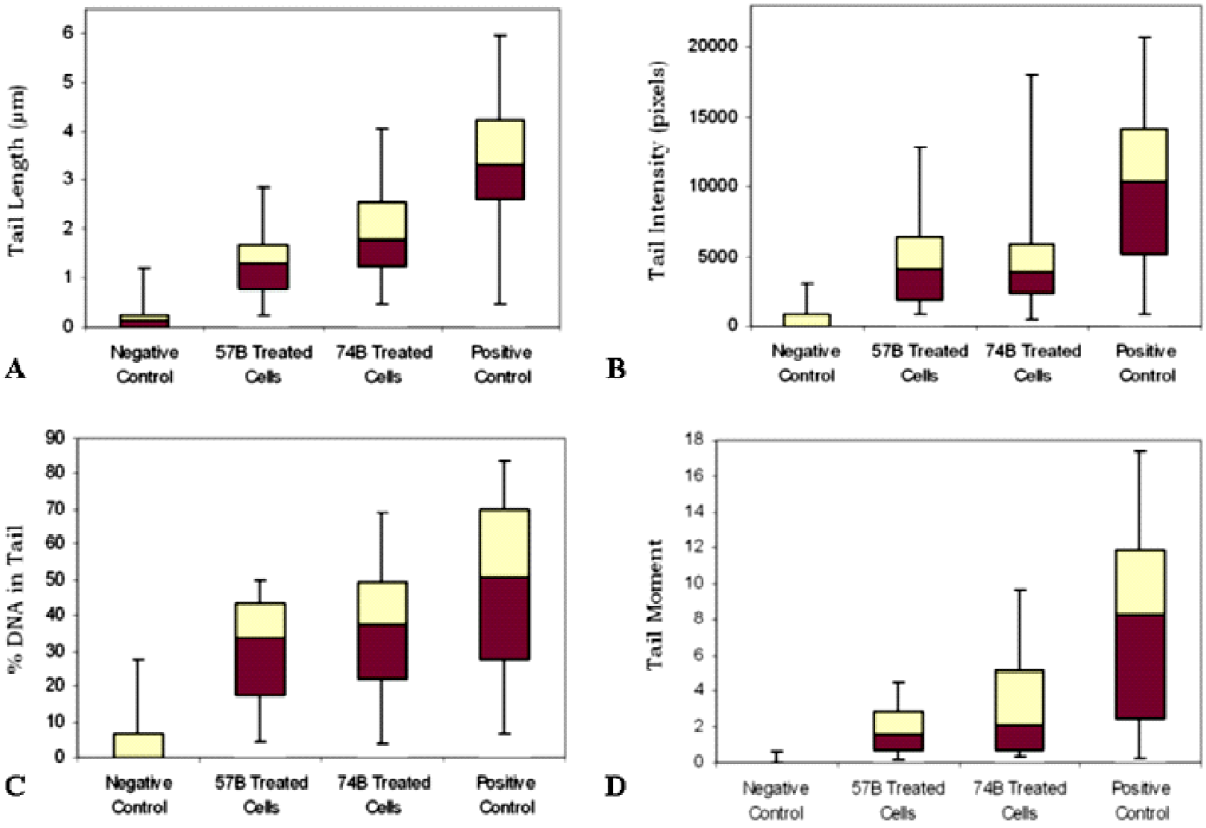


Fig. 7. Box-whisker plot (graphical representation of the smallest observation, lower quartile, median, upper quartile, and largest observation) showing comparison between untreated control, **3n** (200 µg/ml) treated, **3r** (200 µg/ml) treated, and 100 µL hydrogen peroxide treated fungal cells (positive control) for (A) Tail length, (B) Tail intensity, (C) %DNA in tail and (D) Tail moment treated fungal cells.

carboxaldehydes and *o*-arylenediamines in the presence of $FeCl_3$ catalyst and aerial O_2 as the green oxidant. Screening of some selected optically pure benzimidazoles for their antibacterial and antifungal activities using drug resistant clinical isolates was performed, and comet assay of two such selected compounds against *Candida tropicalis* afforded promising results for prospective consideration of these two compounds for dermal application.

Experimental

General:

All commercially available reagents were used directly without further purification unless otherwise stated. All reactions were conducted under atomospheric O_2 (air) unless otherwise stated. Acetonitrile for reaction was dried and distilled prior to use according to standard method (dried over calcium chloride and phosphorus pentachloride). Product purification by column chromatography was accomplished using silica gel (60–120 mesh). Technical grade solvents were used for chromatography and distilled prior to use. Melting points were uncorrected. IR spectra were recorded on BX-II FT-IR spectrometer in KBr with absorptions in wavenumbers (cm^{-1}). ^1H NMR and ^{13}C NMR of the products were determined on Brucker DPX-300 spectrometer at ambient temperature in $CDCl_3$. *J* values are in hertz (Hz). Chemical shifts (δ) are expressed in parts per million (ppm). High Resolution mass spectra (HRMS) were obtained by a Q-tof-Micro (YA-263) mass spectrometer by electron spray ionization method.

Single crystal X-Ray diffraction data were recorded in a Bruker-AXS SMART APEX II X-Ray diffractometer. The cell refinement and data reduction was done using 'Bruker Saint'. Structure refinements and solutions were carried out in SHELXL-97 (Sheldrick, 1997) programme.

*Typical procedure for synthesis of 2-[(2R,3S,4R)-3,4-bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl]-1H-benzo[d]imidazole (**3a**):*

To a round bottom flask **1a** (100.00 mg, 0.225 mmol, 1.0 equiv.), **2a** (36.49 mg, 0.338 mmol, 1.5 equiv.) dry CH_3CN (4 mL) and anhydrous $FeCl_3$ (7.29 mg, 0.045 mmol, 0.2 equiv.) were added sequentially, and then the reaction mixture was refluxed for a certain period (6 h, as monitored by TLC) of time under aerial O_2. After completion of the reaction, the solvent was evaporated under vacuum, and the residue was diluted with CH_2Cl_2. It was washed with aqueous $NaHCO_3$ solution, and the aqueous layer was further extracted with CH_2Cl_2 (3 times). The combined organic layer was washed with H_2O and dried over anhydrous Na_2SO_4. Solvent was removed under rotary evaporator to afford a crude product, which was purified by column chromatography on silica gel using EtOAc-pet. ether (16:84, v/v) as eluent to give the pure product (**3a**, 108 mg) as a white solid in 90% yield. The spectroscopic data and X-ray crystallographic data are in good agreement with the product structure. The same procedure was applied for all the glycal fused chiral benzimidazoles (**3**) (Table 1).

*2-[(2R,3S,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl]-1H-benzo[d]imidazole (**3a**):*

White solid; m.p. 110°C; $[\alpha]_D^{25}$ + 93.7 (*c* 0.33, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3417, 3030, 2863, 1665, 1632,1583, 1517, 1294, 1190, 1097; ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.83 (dd, 1H, *J* 4.2 Hz and 10.7 Hz), 3.91 (dd, 1H, *J* 5.2 Hz and 10.6 Hz), 4.20–4.24 (m, 1H), 4.39–4.44 (m, 1H), 4.50 (d, 1H, *J* 11.4 Hz), 4.60–4.63 (m, 3H), 4.67 (d, 1H, *J* 11.4 Hz), 4.78 (s, 2H), 7.15–7.16 (m, 2H), 7.28–7.29 (m, 2H), 7.32–7.35 (m, 10H), 7.39–7.40 (m, 2H), 7.41–7.42 (m, 3H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ ppm): 67.8, 70.6, 71.9, 72.7, 73.3, 73.6, 76.9, 104.0, 122.1, 127.8, 128.0, 128.1, 128.5, 128.55, 128.6, 128.7, 129.0, 137.3, 137.7, 137.8, 149.2, 150.5; ESI-HR-MS Calcd. for $C_{34}H_{33}N_2O_4$ $[M+H]^+$: 533.2440, Found: 533.2440.

*2-[(2R,3S,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl]-5-chloro-1H-benzo[d]imidazole (**3d**):*

Brown syrup; $[\alpha]_D^{25}$ + 64.6 (*c* 3.1, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3399, 3029, 2865, 1680, 1645, 1570, 1529, 1288, 1189, 1094, 767; ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.82 (dd, 1H, *J* 4.3 Hz and 10.8 Hz), 3.90 (dd, 1H, *J* 5.3 Hz and 10.7 Hz), 4.20 (t, 1H, *J* 5.3 Hz), 4.40–4.50 (m, 2H), 4.57–4.59 (m, 3H), 4.67 (d, 1H, *J* 11.5 Hz), 4.77 (s, 2H), 7.10 (d, 1H, *J* 8.2 Hz), 7.26–7.30 (m, 3H), 7.33–7.34 (m, 10H), 7.39–7.43 (m, 4H), 7.76 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ ppm): 67.7, 70.7, 71.7, 72.6, 72.8, 73.5, 103.7, 122.5, 127.5, 127.8, 127.9, 128.1, 128.5, 128.6, 128.7, 129.0, 137.2, 137.5, 137.7, 149.5, 151.7; ESI-HR-MS Calcd. for $C_{34}H_{32}ClN_2O_4$ $[M+H]^+$: 567.2051, Found: 567.2055.

*2-[(2R,3S,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl)-6-nitro-1H-benzo[d]imidazole (**3e**):*

Dark yellow syrup; $[\alpha]_D^{25}$ + 29.5 (*c* 2.6, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3380, 3089, 3068, 3033, 2924, 2868, 1642, 1519, 1339, 1209, 1094; ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 3.79 (dd, 1H, *J* 4.6 Hz and 10.6 Hz, C_6'-H′), 3.87 (dd, 1H, *J* 5.6 Hz and 10.8 Hz, C_6'-H″), 4.19 (app. t, 1H, *J* 4.4 Hz, 4.8 Hz, C_4'-H), 4.50 (m, 3H, C_5'-H and C_6'-OCH_2Ph), 4.60 (d, *J* 11.2 Hz, 1H, C_3'-H), 4.67–4.74 (m, 4H, C_3'-OCH_2Ph and C_4'-OCH_2Ph), 7.23–7.42 (m, 16H, OCH_2Ph and C_4-H), 8.01 (s, 1H, C_1'-H), 8.04 (dd, 1H, *J* 1.8 Hz, 9.0 Hz, C_5-H), 8.22 (s, 1H, C_7-H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 67.6 (C_6'), 71.1 (-OCH_2Ph), 71.3 (C_4'), 71.9 (C_3'), 72.7(-OCH_2Ph), 73.7 (-OCH_2Ph), 77.1 (C_5'), 102.5 (C_2'), 118.8 (C_5), 128.1 (C_4), 128.4–129.2 (-OCH_2Ph), 137.2 (C_7), 137.4 (C_{7a}), 137.6 (C_{3a}), 143.6 (C_6), 152.6 (C_1'), 154.7 (C_2); ESI-HR-MS Calcd. for $C_{34}H_{32}N_3O_6$ $[M+H]^+$: 578.2291, Found: 578.2286.

*[2-{(2R,3S,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl}-1H-benzo[d]imidazol-6-yl]-phenyl-methanone (**3f**):*

White foamy solid; $[\alpha]_D^{25}$ + 43.2 (*c* 0.37, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3386, 3262, 3029, 2923, 2852, 1644, 1613, 1453, 1296, 1190, 1069; ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.83 (dd, 1H, *J* 4.3 Hz and 10.7 Hz), 3.91 (dd, *J* 5.3 Hz, 10.7 Hz, 1H), 4.22 (t, *J* 5.2 Hz, 1H), 4.44–4.50 (m, 2H), 4.57–4.60 (m, 3H), 4.66–4.70 (m, 1H), 4.77 (s, 2H), 7.27–7.30 (m, 3H), 7.31–7.36 (m, 10H), 7.38–7.39 (m, 3H), 7.46–7.51 (m, 3H), 7.56–7.61 (m, 2H), 7.72 (d, *J* 8.3 Hz, 1H), 7.77–7.80 (m, 1 H), 7.84 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ ppm): 67.7, 70.8, 71.7, 72.6, 72.7, 73.6, 103.7, 124.8, 127.8, 127.9, 128.0, 128.12, 128.14, 128.5, 128.6, 128.8, 129.1, 129.9, 131.5, 131.8, 137.2, 137.5, 137.7, 138.6, 150.2, 196.7; ESI- HR-MS Calcd. for $C_{41}H_{37}N_2O_5$ $[M+H]^+$: 637.2702, Found: 637.2734.

*2-[(2R,3S,4R)-3,4-Dimethoxy-2-(methoxymethyl)-3,4-dihydro-2H-pyran-5-yl)-5,6-dimethyl-1H-benzo[d]imidazole (**3i**):*

Brown syrup; $[\alpha]_D^{25}$ + 3.44 (*c* 2.8, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3409, 3056, 2982, 2957, 2851, 1701, 1645, 1450, 1305, 1271, 1081; ^1H NMR ($CDCl_3$, 500 MHz, δ ppm): 2.31 (s, 6H), 3.40 (s, 3H), 3.50 (s, 3H), 3.57 (s, 3H), 3.65 (dd, 1H, *J* 2.1 Hz, 6.3 Hz), 3.72 (dd, 1H, *J* 3.3 Hz, 6.6 Hz), 3.85 (t, 1H, *J* 3.5 Hz), 4.19–4.21 (m, 1H), 4.59 (d, 1H, *J* 3.0 Hz), 7.34 (s, 2H), 7.78 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz, δ ppm): 20.4, 55.3, 58.7, 59.2, 70.1, 72.9, 74.3, 103.4, 114.5, 132.2, 135.3, 149.4, 150.2; ESI-HR-MS Calcd. for $C_{18}H_{24}N_2O_4Na$ $[M+Na]^+$: 355.1634, Found: 355.1632.

*5-Chloro-2-[(2R,3S,4R)-3,4-dimethoxy-2-(methoxy-methyl)-3,4-dihydro-2H-pyran-5-yl]-1H-benzo[d]imidazole (**3j**):*

Light brown syrup; $[\alpha]_D^{25}$ +18.2 (*c* 1.4, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3051, 2932, 2831, 1647, 1454, 1429, 1281, 1178, 1089; ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 3.39 (s, 3H, C_6'-OMe), 3.47 (s, 3H, C_3'-OMe), 3.54 (s, 3H, C_4'-OMe), 3.63 (dd, 1H, *J* 3.6 Hz and 10.4 Hz, C_6'-H′), 3.71 (dd, 1H, *J* 5.6 Hz and 10.4 Hz, C_6'-H″), 3.83 (app. t, 1H, *J* 4.8 Hz, 6.4 Hz, C_4'-H), 4.20 (dd, 1H, *J* 5.2 Hz and 9.4 Hz, C′-H), 4.50 (d, 1H, *J* 3.9 Hz, C_3'-H), 7.13 (dd, 1H, *J* 1.8 Hz and 8.2 Hz, C_6-H), 7.40 (d, 1H, *J* 8.8 Hz, C_7-H), 7.48 (d, 1H, *J* 1.6 Hz, C_4-H), 7.65 (s, 1H, C_1'-H); ^{13}C NMR ($CDCl_3$, 100 Mz, δ ppm): 55.1 (C_3'-OMe), 58.8 (C_4'-OMe), 59.2 (C_6'-OMe), 70.0 (C_6'), 73.1 (C_4'), 75.1 (C_3'), 77.0 (C_5'), 103.9 (C_2'), 114.5 (C_7), 115.2 (C_4), 122.9 (C_6), 127.9 (C_5), 136.9 (C_{7a}), 139.2 (C_{3a}), 150.0 (C_1'), 151.8 (C_2); ESI-HR-MS Calcd. for $C_{16}H_{20}ClN_2O_4$ $[M+H]^+$: 339.1112, Found: 339.1107.

*2-[(2R,3S,4R)-3,4-Dimethoxy-2-(methoxymethyl)-3,4-dihydro-2H-pyran-5-yl]-6-nitro-1H-benzo[d]imidazole (**3k**):*

Yellow syrup; $[\alpha]_D^{25}$ +9.1 (*c* 1.2 ,$CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3055, 2979, 2839, 1644, 1560, 1454, 1432, 1287, 1182, 1092; ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.4 3 (s, 3H), 3.55 (s, 3H), 3.59 (s, 3H), 3.69 (dd, *J* 3.3 Hz and 10.8 Hz, 1H), 3.77 (dd, *J* 4.9 Hz, 10.8 Hz, 1H), 3.89 (t, *J* 12.5 Hz, 1H), 4.20–4.24 (m, 1H), 4.53 (d, 1H, *J* 5.3 Hz), 7.55 (d, 1H, *J* 8.9 Hz), 7.78 (s, 1H), 8.13 (dd, 1H, *J* 2.1 Hz and 8.8 Hz), 8.43 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ ppm): 55.1, 58.8, 59.2, 69.8, 73.0, 75.3, 103.4, 118.4, 143.3, 151.4, 155.1; ESI-HR-MS Calcd. for $C_{16}H_{19}N_3O_6Na$ $[M+Na]^+$: 372.1172, Found: 372.1172.

*2-[(2R,3R,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl]-5,6-dimethyl-1H-benzo[d]imi-dazole (**3n**):*

Brown syrup; $[\alpha]_D^{27}$+24.3 (*c* 2.28, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3400, 3030, 2922, 2858, 1638, 1454, 1307, 1182, 1090, 1027; ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): 2.31 (s, 6H), 3.88–3.92 (m, 2H), 4.21 (bs, 1H), 4.46–4.59 (m, 3H), 4.65–4.71 (m, 3H), 4.85–4.91 (dd, 2H, *J* 8.2 Hz and 10.2 Hz), 7.14 (bs, 2H), 7.34–7.41 (m, 14H), 7.50 (s, 1H, C_1'-H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ ppm): 20.3, 29.6, 67.7, 71.7,

71.8, 73.1, 73.2, 73.5, 76.1, 104.7, 127.7, 127.9, 128.2, 128.3, 128.4, 128.5, 128.8, 130.8, 137.6, 137.8, 147.6, 149.9; ESI-HR-MS Calcd. for $C_{36}H_{37}N_2O_4$ $[M+H]^+$: 561.2753, Found: 561.2734.

*2-[(2R,3R,4R)-3,4-Bis(benzyloxy)-2-(benzyloxymethyl)-3,4-dihydro-2H-pyran-5-yl]-6-nitro-1H-benzo[d]imidazole (**3o**):*

Yellow syrup; $[\alpha]_D^{25}$ +104.4 (*c* 1.9, $CHCl_3$); IR (KBr, ν_{max}/cm^{-1}): 3375, 3088, 3063, 3030, 2921, 2866, 1640, 1516, 1338, 1207, 1091; ^1H NMR ($CDCl_3$, 500 MHz, δ): 3.79–3.80 (m, 2H), 4.18 (s, 1H), 4.37 (s, 1H), 4.42 (d, 1H, *J* 6.9 Hz), 4.49 (d, 1H, *J* 6.9 Hz), 4.58 (d, 1H, *J* 6.9 Hz), 4.68 (d, 2H, *J* 6.9 Hz), 4.81 (m, 2H), 7.17 (s, 1H), 7.23–7.32 (m, 12H), 7.39–7.40 (m, 3H), 7.65 (s, 1H), 8.00 (d, 1H, *J* 6.9 Hz), 8.10 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz, δ): 67.7, 70.7, 72.6, 73.2, 73.8, 74.1, 103.7, 118.3, 128.0, 128.1, 128.2, 128.66, 128.68, 129.1, 129.4, 137.2, 137.7, 137.8, 143.2, 150.7; ESI-HR-MS Calcd. for $C_{34}H_{32}N_3O_6$ $[M+H]^+$: 578.2291, Found: 578.2286.

*2-[(2R,3R,4R)-3,4-Dimethoxy-2-(methoxymethyl)-3,4-dihydro-2H-pyran-5-yl]-1H-anthra[1,2-d]imidazole-6,11-dione (**3r**)*:

Orange solid; m.p. 236°C; IR (KBr, ν_{max}/cm^{-1}): 3440, 3079, 2920, 2842, 1730, 1631, 1570, 1442, 1280, 1186, 1050; ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 3.46 (s, 3H, C_6'-OMe), 3.70 (s, 3H, C_4'-OMe), 3.76 (d, 2H, *J* 6.4 Hz, C_6'-H′H″), 3.87 (s, 3H, C_3'-OMe), 4.03 (app. t, 1H, *J* 1.2 Hz and 2.0 Hz, C_4'-H), 4.35 (t, 1H, *J* 6.4 Hz, C_5'-H), 4.60 (d, 1H, *J* 2.4 Hz, C_3'-H), 7.72–7.78 (m, 2H, C_8-H and C_9-H), 7.86 (s, 1H, C_1'-H), 7.93 (d, 1H, *J* 8.4 Hz, C_4-H), 8.15 (d, 1H, *J* 8.4 Hz, C_5-H), 8.23 (dd, 1H, *J* 2.0 HZ and 5.6 Hz, C_7), 8.28 (dd, 1H, *J* 1.6 Hz and 7.2 Hz, C_{10}-H), 11.58 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 58.1 (C_3'-OMe), 59.4 (C_6'-OMe), 60.8 (C_4'-OMe), 70,0 (C_6'), 71.2 (C_4'), 74.9 (C_3'), 77.3 (C_5'), 103.4 (C_2'), 117.6 (C_{11a}), 122.1 (C_5), 123.8 (C_4), 126.5 (C_7), 127.6 (C_{5a}), 127.7 (C_{10}), 132.3 (C_{11b}), 133.4 (C_{10a}), 133.7 (C_8), 134.1 (C_{6a}), 134.3 (C_9), 148.2 (C_{3a}), 151.5 (C_2'), 156.5 (C_2), 182.7 (C_{11}), 184.9 (C_6); ESI-HR-MS Calcd. for $C_{24}H_{23}N_2O_6$ $[M+H]^+$: 435.1556, Found 435.1550.

Antimicrobial activity:

Microorganisms for study: A total of ten microbial cultures, comprising eight bacterial and two fungal strains, were collected from superficial and subcutaneous infections for this study. All the bacterial and fungal isolates, including MSSA (methicillin sensitive *Staphylococcus aureus*), MRSA (methicillin resistance *S. aureus*), and ESBL (extended spectrum β-lactamase) – producer strains, were charecterized by standard laboratory protocol[21–23].

Bacterial strains: Seven synthesized benzimidazole derivatives (**3a**, **3c**, **3g**, **3l**, **3n**, **3o**, **3r**) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4 mg/mL, and screened for antibacterial activity by the agar well diffusion assay[18]. The bacterial culture in Muller Hinton broth (MHB) was adjusted to the final inoculum density of 1×10^7 cfu/mL (by 0.5 McFarland standard) on molten Muller Hinton agar (MHA) plates. After solidification, wells (diameter 5 mm) were made with a sterile borer in the inoculated MHA plates. 50 μL solution containing 0.2 mg of each compound was dispensed in each well, while DMSO was also tested as the vehicle control. Ciprofloxacin and gentamicin were the standard drugs used as positive controls in this assay. Antibacterial activity was expressed as the diameters of inhibition zones produced by the antimicrobials, and measured after 24 h of incubation at 37°C.

Fungal strains: The selected benzimidazole derivatives were screened for antifungal activity[18]. Fungal culture in Sabouraud Dextrose broth (SDB) containing an inoculum density of 0.5 McFarland (1×10^8 cfu/mL) was diluted at 1:10 ratio in SDA plate to obtain the final inoculum concentration of 1×10^7 cfu/mL. Wells (diameter 5 mm) were punched on solidified SDA plates and 50 μL solution containing 0.2 mg of each compound was dispensed in each well, and DMSO was tested as the vehicle control. Fluconazole (0.02 mg/well; dissolved in DMSO) was used alone as a standard antifungal agent. The diameter of the inhibition zone was measured after 24 h of incubation at 35°C. Antifungal activity was expressed as diameters of inhibition zones around the wells produced by the tested compounds and antifungal agent.

Determination of minimum inhibitory concentration (MIC):

Bacterial strains: The MIC of the benzimidazole derivatives against the tested bacteria was determined by broth micro-dilution procedure to find the lowest concentration of the extract at which no growth was visible. Stock solutions (1.024 mg/mL) of seven benzimidazole derivatives were prepared in DMSO, and serially diluted in MHB at concentrations of 512, 256, 128, 64, 32, 16, 8 and 4 μg/mL in a 96-well microtitre plate. The broth culture containing 0.5 McFarland (1×10^8 cfu/mL) inoculum density was then introduced to each

of the microtitre wells at 1:10 ratio to maintain final inoculum density of 1×10^7 cfu/mL. Microtitre plates were incubated for 18 h at 37°C, and the presence of visible growth in each well was inferred by measuring OD at 630 nm using ELISA reader (Erba LisaScan II, Transasia)[18,21].

Fungal strains: MIC for benzimidazole derivatives against the fungal isolates was determined in a similar procedure, using SDB in 96-well microtitre plates incubated for 18 h at 35°C[24]. The presence of visible growth was determined spectrophotometrically as above.

Determination of drug resistance profile of the isolated bacteria: Antibacterial susceptibility studies were carried out by Kirby and Bauer disk diffusion technique using commercially available antibiotic disks. Bacterial culture in peptone water (Hi-Media, India) containing 0.5 McFarland turbidity (1×10^8 cfu/mL) was swabbed in MHA plate. Antibiotic disks were placed on it by maintaining about 20 mm distance with each other[21,25]. Inhibition zone diameter was measured after overnight incubation at 37°C and results were interpreted as per CLSI guidelines[21].

Evaluation of benzimidazole derivative induced DNA damage in Candida tropicalis by-alkaline single cell gel electrophoresis (SCGE, or Comet assay): Candida tropicalis cell suspension at 0.5 McFarland (~ 1×10^8 Cells/ml) was diluted in nuclease free water (to prepare negative control), 100 μM H_2O_2 solution (to prepare positive control), **3n** and **3r** (200 μg/mL) solution at a ratio of 1:10 (v/v) and kept for 30 min at 4°C. After that, aliquots from negative control, positive control, **3n** and **3r** treated samples were further diluted in phosphate buffered saline (PBS; Ca^{2+} and Mg^{2+} free; pH 7.4) at a ratio of 1:100 (v/v) to attain final cell density 1×10^5/mL. SCGE was performed using comet assay reagent kit (Trivigen, Gaithersburg, MD, catalog # 4250-050-K) according to the manufacturer's instructions. Briefly, *C. tropicalis* cell suspension (1×10^5 cells/ml) in PBS was combined with 1% molten low melting agarose (Catalog # 4250-050-02) at 37°C at a ratio of 1:10 (v/v) and transferred to comet slide (Catalog # 4250-050-03). After gelling time (~30 min), the slide was immersed respectively, in pre-chilled (4°C) lysis solution (Catalog # 4250-050-01) and alkaline solution (200 mM NaOH, 1 mM EDTA, pH > 13) for 45 min in each. Then electrophoresis of slide was conducted in pre-chilled (4°C) alkaline electrophoresis[26] solution (200 mM NaOH, 1 mM EDTA, pH > 13) for 30 min at 25 volt and 300 mA (1.5 V/cm), and then washed in deionized water, dehydrated in 70% ethanol and stained with SYBR® Green I (Catalog # 4250-050-05, maximum excitation/emission was 494 nm/521 nm) diluted in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA)[27]. Fluorescent comet patterns were examined with a MOTIC BA400 microscope under 200× magnification and fluoro-isothiocyanate (FITC) filter combination and images captured by a charge-couple device (CCD) camera.

Image analysis: Comet Score Freeware v1.5 (TriTek Corporation) was used to measure comet length (number of pixels in the horizontal direction of the comet), Comet Height (pixels in the vertical direction of the comet), Comet Area (number of pixels in the comet), Comet Intensity (sum of all pixel intensity values in the comet), Comet Mean Intensity (average of all pixel intensity values in the comet), Head Diameter (number of pixels in horizontal direction of the comet head), Head Area (number of pixels in comet head), Head Mean Intensity (average of all pixel intensity values in the comet head), %DNA in Head (total comet head intensity divided by the total comet intensity, multiplied by 100), Tail Length (comet head diameter subtracted from the overall comet length), Tail Area (number of pixels in the comet tail), Tail Intensity (sum of all pixel intensity values in comet tail), %DNA in Tail (total comet tail intensity divided by the total comet intensity, multiplied by 100), Tail Moment (%DNA in the comet tail multiplied by the tail length), and Olive Moment[28] (summation of each tail intensity integral value, multiplied by its relative distance from the center of the head and divided by the total comet intensity)[27], Hundred cells per sample were observed using this software.

Results were expressed as means ± SE and statistical comparison of results with negative control sample value, were performed by independent, two tailed, Student's t-test. Differences were considered significant at $p < 0.05$. Comparison of tail length, tail intensity, %DNA in tail and tail moment within different samples was depicted using box-whisker plot (done by Microsoft Excel 2002 statistical software).

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