Chapter 4

Chemical modification of isolated metabolites and cytotoxic evaluation

4. Chemical modification of isolated metabolites and cytotoxic evaluation

4.1. Introduction

Currently, a large number of triterpenoid drugs are present in the market and used for the treatment of various diseases. They exhibit a range of pharmacological activities, including anti-inflammatory, hepatoprotective, and anticancer properties (Figure 4.1 a and b) [95-98]. Drugs containing triterpenoids skeleton like ursodiol (ursolic acid derivative) is used clinically to treat gallstones and certain liver diseases [99], betulinic acid derivatives have been investigated for their potential in treating melanoma, lung cancer, and other malignancies [100]. Various derivatives of artemisinin, such as artesunate and artemether, are used in combination therapies for the treatment of malaria, particularly in regions where resistance to other antimalarial drugs is prevalent [101]. Triterpenoids, like boswellic acids, are known for their anti-inflammatory and analgesic properties. Boswellic acid analogues are marketed as dietary supplements for managing osteoarthritis and other inflammatory conditions [102, 103]. Also, triterpenoid, saponin, and glycyrrhizin possess anti-inflammatory, antiviral, and hepatoprotective properties [104]. It is used clinically in the treatment of chronic hepatitis and as an adjunct therapy for conditions such as peptic ulcers. Moreover, in 2023, three new drugs named celastrol, vamorolone, and omaveloxolone containing triterpenoid skeleton were approved by the FDA for the treatment of the autoimmune disorder Duchenne muscular dystrophy and Friedrich ataxia (Figure 4.1 c) [105-107]. Also, various triterpenoids have demonstrated cytotoxic and promising anticancer properties in preclinical animal studies. Researchers have expanded this potential by inducing chemical modification in the naturally occurring triterpenoids. The isolation chemistry conducted on a cytotoxic natural product lead typically yields a spectrum of structurally related active compounds. This process enables an initial assessment of the relationships between molecular structure and cytotoxic

activity [108]. Such insights serve as a foundation for guiding the synthesis of enhanced analogues of the bioactive compound with potentially greater efficacy. For the same, various chemical functionalities are incorporated into the lead structure.

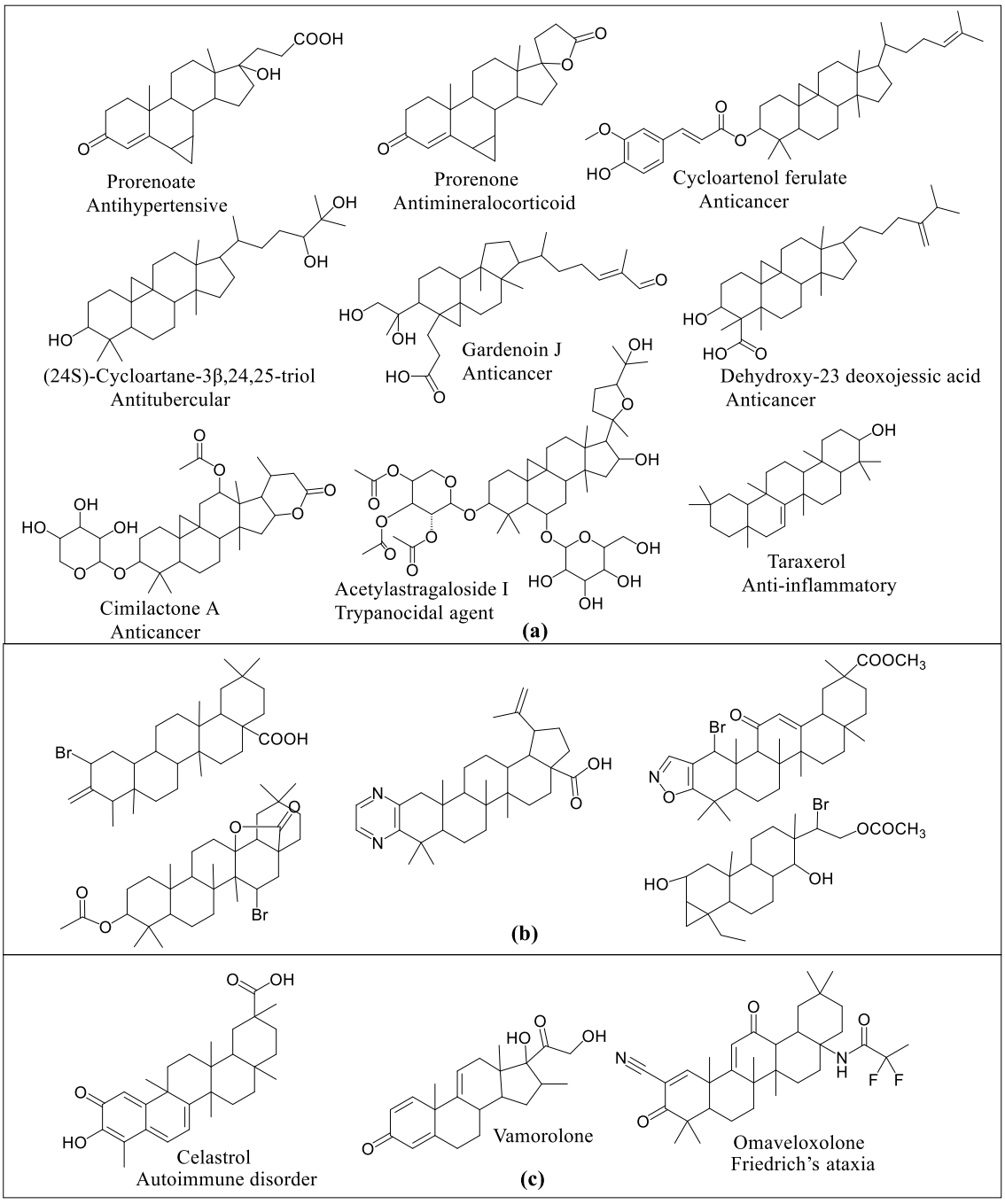


Figure 4.1. Semi-synthetically modified triterpenoids (a) Marketed drugs (b) Cytotoxic agents under preclinical studies and (c) FDA-approved drugs in 2023

Nitrogenous compounds play a crucial role in drug discovery due to their diverse chemical properties and biological activities [109]. Nitrogen can form various chemical

bonds, and this versatility allows nitrogen-containing compounds to exhibit diverse chemical structures that can be tailored to specific biological pathways and molecular targets [110]. Nitrogen-containing functional groups, such as amines, amides, azides, and heterocycles like indole, pyrimidine, pyrazine, etc, often contribute to the pharmacological properties of drugs by interacting with receptors, enzymes, and other biomolecules in the body [111]. These compounds can be designed to selectively target specific biological pathways implicated in disease. Through rational drug design and structure-activity relationship studies, nitrogen-containing drugs can be optimized for high affinity and selectivity toward their intended molecular targets, reducing off-target effects and improving therapeutic outcomes [112]. For example, the presence of basic nitrogen atoms in a molecule can enhance its solubility, membrane permeability, and metabolic stability, leading to improved bioavailability and prolonged systemic exposure[113]. Moreover, nitrogenous compounds are often readily accessible through synthetic routes, facilitating their synthesis and optimization in drug discovery programs. Advances in organic synthesis methodologies have enabled the efficient synthesis of nitrogen-containing scaffolds and the introduction of functional groups, allowing for the synthesis of diverse chemical libraries for screening against biological targets. Nitrogenous compounds are indispensable in drug discovery due to their structural diversity, biological activity, target specificity, pharmacokinetic properties, synthetic accessibility, and potential to overcome drug resistance [114, 115].

Halogenation is another important reaction in organic chemistry. It opens a gateway to further chemical transformation. The free radical halogenation of alkanes entails the replacement of a hydrogen atom within the alkane molecule with a halogen atom, resulting in the formation of a haloalkane [116, 117]. These functional groups serve as important intermediates in various organic synthesis pathways, enabling the preparation

of diverse organic compounds. Introducing halogen atoms into organic molecules alters their reactivity and chemical behaviour. Halogen substituents can influence the stability, polarity, and electronic properties of organic compounds, affecting their reactivity in subsequent reactions [118]. Synthesis of halogenated aromatic compounds can be done using organic, inorganic, and electrochemical methods. During organic synthesis, N-halo succinimides (NXS X = Cl, NCS; Br, NBS; and I, NIS) have been directly used as halogenating reagents to avoid toxic Cl_2 or Br_2 [119-121]. The success of NXS as the halogenating reagent largely depends on the catalyst, nature of the substrate, reaction conditions, and solvent.

Therefore, our next objective was to introduce chemical modification in the isolated triterpenoids from *D.malabaricum* by synthesizing the nitrogenous derivative of beddomeilactone in one of the series and bromination of beddomeilactone using N-bromosuccinamide to functionalize aliphatic side chain for further reaction in another series.

4.2. Rationale designed

The new compounds were isolated in less amount (20-30 mg) while beddomeilactone (4gm) and dihydrobeddomeilactone (3gm) were obtained in good amounts. Beddomeilactone exhibits promising biological activity that is antimicrobial, antimalarial, and anti-inflammatory. Despite its potential, there is an absence of reported studies on its synthetic modification, hindering further exploration of its pharmacological properties. In response to this gap, we propose a chemical modification strategy for beddomeilactone to enhance potency and introduce functionalization in the cycloartane triterpenoids skeleton. This study outlines the rational design and synthetic route for modifying beddomeilactone, incorporating organic synthesis techniques and structural elucidation methods. The proposed chemical modification of beddomeilactone involved

key transformations such as functional group reaction, stereochemical modifications, and structural diversifications. The synthetic strategy incorporates transition metal-catalyzed reactions and heterocyclic chemistry at three reactive sites of beddomeilactone. These reactive sites are shown in Figure 4.2.

1. Substitution at carbonyl carbon present in the aliphatic side chain
2. Substitution at alpha carbon of carbonyl group present in the aliphatic side chain.
3. Substitution at ring A in acid functionality.

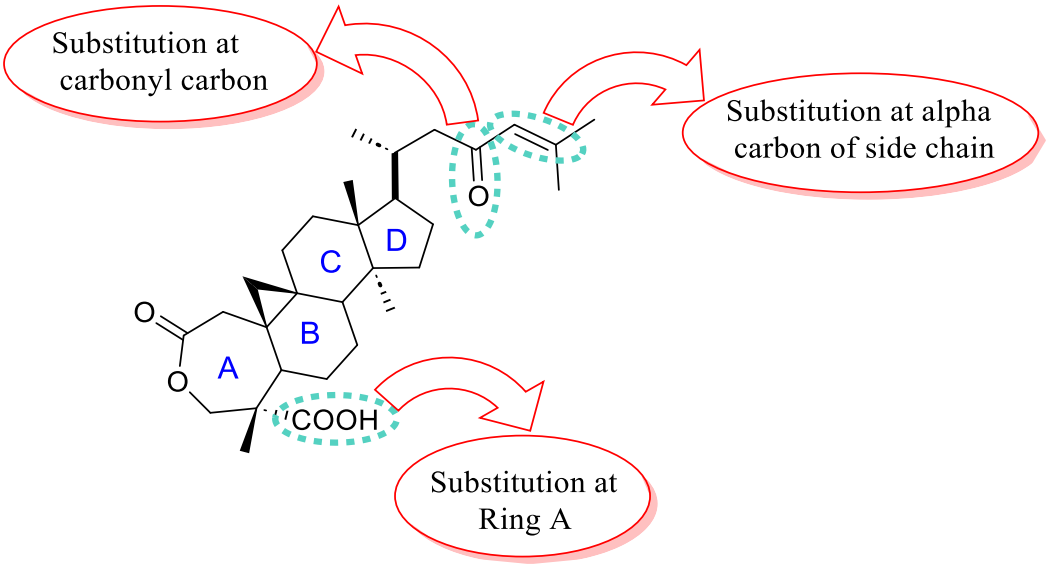


Figure 4.2. Reactive sites of beddomeilactone

Upon successful implementation of the proposed synthetic modifications, different derivatives of beddomeilactone were synthesized and evaluated for their cytotoxic potential.

4.3. Experimental section

4.3.1. General experimental procedure

All chemicals were obtained from Sigma-Aldrich Company and used as received. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker-Avance III HD 500 MHz NMR spectrometer using tetramethylsilane (TMS) as the internal standard and are referenced to the residual proton/carbon in the NMR solvent ($CDCl_3$, 7.26/77.1 ppm; CD_3OD, 4.89,

3.30/49.0 ppm, DMSO-d_6, 2.50/39.5 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-QTOF and HRMS-6540-UHD spectrometers.

4.3.2. General method of amide synthesis

To the mixture of aliphatic/aromatic carboxylic acid (1 equiv) and phenylhydrazine (1.5 equiv.), zinc chloride (15 mol%) was added in dioxane, and the reaction mixture was heated at 120 °C for 12 h. under reflux. The reaction was monitored by TLC. After the reaction (usually 12 h) was completed, the reaction mixture was filtered, concentrated and partitioned in ethyl acetate and bicarbonated water. The organic layer was dried over anhydrous sodium sulphate and concentrated. The amide products were obtained via purification on silica gel column chromatography.

4.3.3. General method of halogenation reactions

One mmol of substrate was dissolved in methanol and water mixture and added to a solution of equivalent amount of NXS and 10 wt % of catalyst. The reaction mixture was carried out for 1 h at room temperature. The catalyst was separated, and the products were isolated by evaporating methanol and extracted with diethyl ether for GCMS analysis. If required, the product was purified over a silica gel column

4.3.4. General procedure of oxidative esterification

To a solution of aldehyde (1 mmol) in corresponding alcohol (2 mL) or thiol (0.5 ml) was added NBS (0.25 equiv). The reaction mixture was stirred for a required period of time, and after completion of the reaction, the product mixture was concentrated, and the reaction mixture was separated by portioning in ethyl acetate-water mixture; the organic layer was concentrated and analyzed by GCMS or MS. Finally, compounds were purified by silica gel column chromatography.

4.4. Result and Discussion

4.4.1. Series 1: Synthesis of nitrogenous derivatives

Numerous reactions were used during our attempts to synthesize nitrogenous compounds, but various challenges were encountered in obtaining significant yields of desired products. Despite the synthesis of various nitrogen-containing compounds, the desired products were not always obtained, and in some instances, product formation did not occur. In one of our studies, the derivatization of beddomeilactone (BML) at the ketone functionality present at the aliphatic side chain was planned to create various indole derivatives by reported methods of Fischer indole synthesis (Figure 4.3) using $ZnCl_2$ as lewis acid. While producing indole derivatives, amide products were inadvertently synthesized instead of indole and the study reports a $ZnCl_2$-catalyzed amide bond formation directly from carboxylic acids and hydrazines.

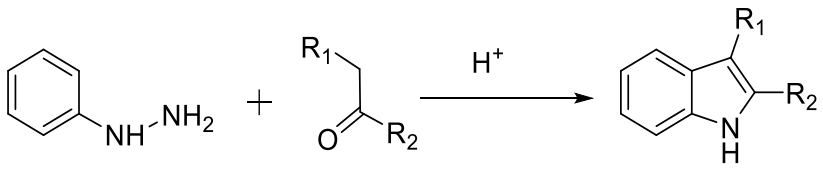


Figure 4.3. Scheme representing Fisher indole synthesis.

The unexpected result was observed when the reaction of BML with phenylhydrazine was carried out in the presence of $ZnCl_2$ under heating and acidic conditions using acetic acid. Unpredictably, the reaction produced amides, and the desired product was not observed in this reaction (Figure 4.4). Exploring this reaction further, it was discovered that phenylhydrazine when reacts with acetic acid in the presence of $ZnCl_2$ under heating, produces the corresponding N-phenylacetamide products in excellent yield.

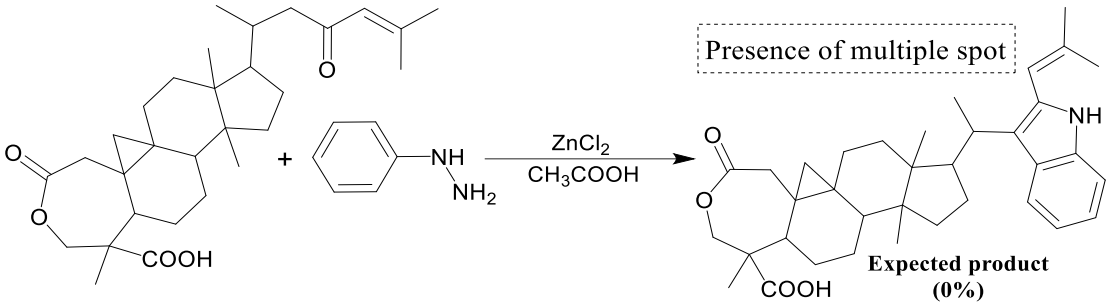


Figure 4.4. Reaction of beddomeilactone and phenylhydrazine.

4.4.1.1. Optimization of reaction condition for amide synthesis

Considering the novelty and importance of this observation, the scope of this reaction was explored in detail using different solvents and reaction conditions. However, phenylhydrazine as an amine surrogate for amide synthesis has never been explored before. This is the first report of amide synthesis directly from carboxylic acid with hydrazine under the optimized reaction condition. To identify the best-optimized state, the study was initiated with a preliminary reaction of phenylhydrazine with acetic acid in the presence of 5 mol% $ZnCl_2$ under an inert and neat medium for 12-48 h at room temperature; the reaction did not produce the corresponding amide product (entries 2 and 3 of Table 4.1). Amide, N-phenylacetamide (1c) was observed when the same reaction was carried out at an elevated temperature of 120 °C without an inert environment (entry 5 of Table 4.1). The product yield was further improved when the catalyst amount was increased to 15 mol%. It was noticed that 15 mol% was the optimum amount (Figure 4.5) and further increments (up to 100 mol%) did not significantly change the yield (entry 6 of Table 4.1)**.**

It was observed that the reaction proceeded without solvent when used with acid in a liquid state like acetic acid and failed to produce the corresponding amide when used for solid acids such as benzoic acid. Next, the objective was to optimize a suitable solvent for the reaction. The reaction either failed or produced less yield with a low boiling point, aprotic solvents such as chloroform, dichloromethane, and acetonitrile even after

prolonged heating for 24 h (entries 7-9 of Table 1). The yield of the product improved marginally in THF when heated to the boiling point for a longer time, 25 h (entry 10 of Table 1). Product yield was significantly enhanced in high boiling point solvents xylene, toluene, and dioxane. Dioxane was found to be most suitable for the reaction (entries 11-13 of Table 4.1). Comparatively, the poor yield was noticed in methanol and DMF (entries 14-15 of Table 4.1) and reaction in the semi-aqueous; methanol-water (9:1) gave a poor outcome (40%), but it tolerates the aqueous solvents. The reaction in an airtight, closed inert (argon flushed) vessel either was unable to give a product or led to inferior yields (<10%), possibly either the production of gaseous side products (N_2 or NH_3) or environmental oxygen plays role in the reaction.

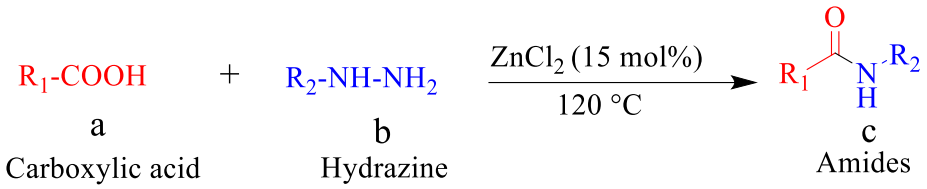


Figure 4.5. Optimized reaction condition of amide synthesis.

Table 4.1. Optimization of reaction condition for amide synthesis.

Entry	Catalyst (mol%)	Solvent	Temp(°C)/Time (h)	Yield[a]
1	None	None	150/ 48	0
2	$ZnCl_2$ (5)	None	25/ 48	0
3	$ZnCl_2$ (5)	None	37/48	<10[b]
4	$ZnCl_2$ (5)	None	120/12	35[b]
5	$ZnCl_2$ (15)	None	120/12	70
6	$ZnCl_2$ (100)	None	120/12	70
7	$ZnCl_2$ (15)	$CHCl_3$	50/24	<10
8	$ZnCl_2$ (15)	DCM	50/24	<10
9	$ZnCl_2$ (15)	ACN	60/24	<10
10	$ZnCl_2$ (15)	THF	75/25	30
11	$ZnCl_2$ (15)	Xylene	120/12	70
12	$ZnCl_2$ (15)	Toluene	120/12	72
13	**$ZnCl_2$ (15)**	**Dioxane**	**120/12**	**90**
14	$ZnCl_2$ (15)	MeOH	60/24	35[c]
15	$ZnCl_2$ (15)	DMF	60/24	35
16	$ZnCl_2$ (15)	MeOH-H_2O (9:1)	60/24	40
17	$ZnCl_2$ (15)	Dry dioxane	120/12	<5[d]

[a] isolated yields; [b] under inert conditions by argon flushing, dry solvents. [c] Similar results were observed with EtOH. [d]Dry dioxane, argon flushed, airtight vessel.

4.4.1.2. Synthesis of amide derivatives

The reaction was not further investigated in detail for the impact of additives as the reaction appears novel, and the product yield is moderate to high. The objective of the optimized reaction was to investigate the scope of the reaction for a wide variety of reactants. The scope of this reaction was then explored for different carboxylic acids using optimized reaction conditions (entry 13 of Table 4.1). Various carboxylic acids and hydrazines were investigated for amide bond formation. First, the reaction was investigated for various aliphatic carboxylic acids, as shown in Figure 4.6. The reaction

of phenylhydrazine, 4-chloro-phenylhydrazine and 4-bromo-phenylhydrazine reacted smoothly with acetic acid to give the corresponding phenylacetamides (1c-3c) in excellent yields (>88%). Further, the scope of the reaction was extended to long-chain fatty acids. The reaction proceeded very well with long-chain fatty acids. Saturated fatty acid; stearic acid reacted smoothly with the phenylhydrazine, 4-chloro-phenylhydrazine and 4-bromo-phenylhydrazine to produce corresponding phenylalkylamide (4c-6c) in 82-90% yield under the optimized reaction condition. Similar results were obtained with unsaturated long-chain fatty acid; oleic acid reacted with phenylhydrazine and 4-bromo-phenylhydrazine (7c and 8c). These examples revealed that the reaction tolerates the double bond and halogen functionality. Further, the reaction of oleic acid with 2,4-dinitrophenylhydrazine under the standard optimized condition produced N-(2-amino-4-nitrophenyl)-oleamide (9c) with moderate yield (64%). It may be because of the partial reduction of one nitro group to amino. Perhaps $ZnCl_2$ also catalyzes the reduction of nitro to amino; hence, it may undergo reduction and amidation simultaneously. The aliphatic and aromatic carboxylic acids underwent amide bond formation with various phenylhydrazines very well. The reaction of benzoic acid with phenylhydrazine, 4-chloro-phenylhydrazine and 4-bromo-phenylhydrazine produced corresponding phenylbenzamides (10c-12c) in 88-91% yield. Cinnamic acid underwent amide synthesis with phenylhydrazine and 4-chlorophenylhydrazine, and the corresponding amide (13c and 14c) was obtained with 85-86% yield. Similar results were obtained when phenylacetic acid reacted with phenylhydrazine to give 15c. Further, the reaction scope was extended to carboxylic acid substrates, which carry additional reactive functional groups such as caffeic acid, 4-aminobenzoic acid (PABA), and 3-chloro-4-nitro benzoic acid. Caffeic acid and PABA reacted with phenylhydrazine very well to yield the amide product 16c and 17c, respectively, with good yield (approx. 75%). This also revealed that

the reaction tolerates the phenolic OH and NH_2 functionality. The reaction of 3-chloro-4-nitrobenzoic acid with phenylhydrazine yielded the corresponding amide 18c with a lower yield (64%). A few additional spots of minor products were observed in this reaction on the TLC. Perhaps the nitro group interferes in the reaction, and reduction of NO_2 to NH_2 might have occurred, as observed in the example of the synthesis of 9c. Further, amidation was carried out on amino acids to generalize this method. Glycine was taken to react with 4-chlorophenylhydrazine. Glycine was not soluble in dioxane; hence, the reaction was carried out in a mixture of methanol in dioxane (2:8). Excess of 4-chlorophenylhydrazine (3 equiv) was required as we experienced the formation of the side product. As expected, glycine-anilide 19c was obtained with 70% yield along with 19 d, which is the product of the side reaction of methanol with 4-chlorophenylhydrazine. The purification of amino acid was difficult on the regular silica gel column, so glycine-anilide 19c and 19d were characterized in the reaction mixture. Also, phenylhydrazine substituted with an electron donating group when reacted with acids resulted in the formation of amides having more than 70% yield. 4-methoxyphenylhydrazine and 4-ethoxyphenylhydrazine give corresponding amides in reaction with benzoic acid and 2-Flurobenzoic acid (20c-23c). Also, the alkyl-substituted hydrazine-like butylhydrazine in reaction with benzoic acid and 4-chlorobenzoic acid gives a good yield of 24c (85%) and 25c (80%). Furthermore, the application of the developed protocol of amide bond formation was extended to complex molecules like natural products and drugs that carry free carboxylic acid functionality for their derivative synthesis. A natural product, 3-acetyl-11-keto-beta-boswellic acid, was subjected to react with phenylhydrazine and 4-chloro-phenylhydrazine under the optimized reaction condition. The desirable amide derivatives (26c and 27c) were obtained in good yield (≥75%). Similarly, antibiotic ciprofloxacin was subjected to amidation, and as expected, the desired amide product 28c

was obtained with a 62% yield. The reaction condition tolerates the labile carbonyl functional group (11-keto functionality in boswellic acid) and the secondary amine group, as revealed in these examples. This protocol was used to prepare dimethylformamide (29c, DMF) by utilizing formic acid and N, N-dimethylhydrazine. However, the condition was modified with reaction parameters. The reaction was carried out for a longer duration (48 h) at a lower temperature (70°C) as methanol is a low boiling point solvent. The reaction was carried out in a dioxane-methanol mixture (9.5:0.5), and DMF formation was observed.

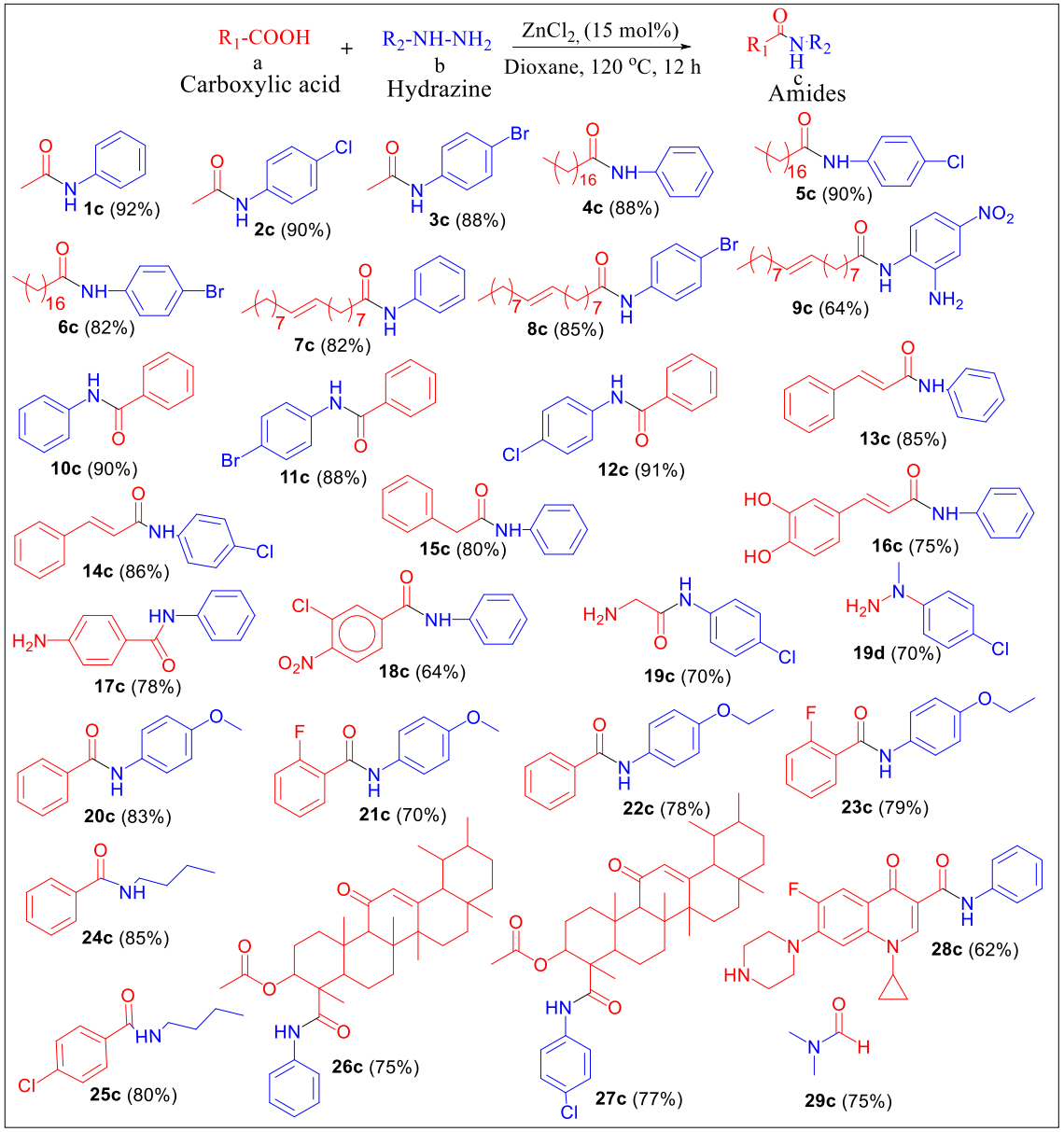


Figure 4.6. Synthesized amide derivatives.

Moreover, to confirm the reaction applicability in synthetic chemistry, the feasibility of scaling up the reaction was examined. The reactions were scaled up for synthesis of 1c, 7c, and 29c, and it went smoothly, as illustrated in Figure 4.3, producing 1.3gm, 1.0 gm, and 5 ml of each product, respectively. Therefore, a new methodology for scale-up synthesis was developed here, allowing gram quantity synthesis of different amides. However, several parameters must be recalculated to scale the reaction, including the amount of $ZnCl_2$. Again, optimizing other parameters for scaling up was beyond the scope of current work and may be subjected to further investigation. The reaction protocol provides an alternative process for making commercially valuable products.

4.4.1.3. Plausible reaction mechanism of amide synthesis

The plausible mechanism in this amidation reaction involves the critical event of rupture of the N-N bond of phenylhydrazine. Both radical, radical ion, and ionic mechanisms report the N-N bond cleavage. The cleavage of the N-N bond is often accomplished by reductive, oxidative, photocatalytic, and other methods. A few control experiments were performed to elucidate the mechanism. The reaction of phenylhydrazine with benzoic acid underwent in the presence of radical scavenger; 2,2,6,6-tetramethylpiperidine-1-yl)oxyl (TEMPO); however, it slowed down the rate of the reaction and the yield of the product was slightly lower (60%), here the possibility of radical mechanism cannot be dismissed. Poor conversion to the desired amide was noticed when the reaction was conducted under a dry, moisture-free, inert, and sealed environment. This result hinted that oxygen also plays an important role in rupturing the N–N bond. Hydrazines are the ambient nucleophile [122]. Both nitrogen act as a nucleophile and in the case of asymmetrical hydrazine; like phenylhydrazines, the substituted nitrogen is a more reactive nucleophile. In phenylhydrazine, the phenyl-substituted nitrogen deprotonates first and generates nucleophiles. The resulting nucleophile attacks the carbonyl

functionality of carboxylic acids [122-124]. The progress of the reaction on TLC was monitored and an intense new spot appeared after five hours of the reaction which disappeared after the completion of the reaction. To identify this intermediate, an aliquot of the reaction was taken after 5.5 h of the reaction progress, and it was loaded to preparative TLC to purify this intermediate immediately. The obtained intermediate was then characterized as hydrazide (30), as shown in Figure 4.7.

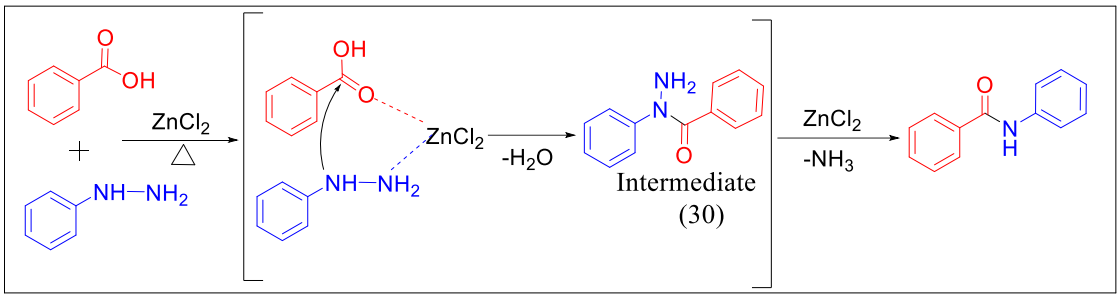


Figure 4.7. Plausible mechanism of amide synthesis.

The reaction undergoes two major events *viz*. [1]; Generation of nitrogen nucleophile, nucleophilic attack on carboxylic acid and formation of intermediate and [2]; N-N bond cleavage of intermediate coordinates both processes. Such deamination usually releases nitrogen in the form of ammonia or N_2, and the formation of ammonia in this process was noticed. The released ammonia was determined by performing a trap experiment described in Figure 4.8. Ammonia was trapped in the trap solution containing a mixture of phenylthioisocynate (PITC) and pyridine in methanol. It was observed that the trapped ammonia results in the formation of 1-phenylthiourea *via* reacting with PITC. This was confirmed by co-TLC spot with standard and NMR spectra. N-N bond cleavage is reported *via* radical, ionic, and radical-ion pathways. Here, it was difficult to conclude the exact path of the reaction. Still, the N-N bond cleavage of the intermediate may proceed through the path incorporating initial complexation with zinc ion, which may follow either ionic or radical cleavage pathway [125].

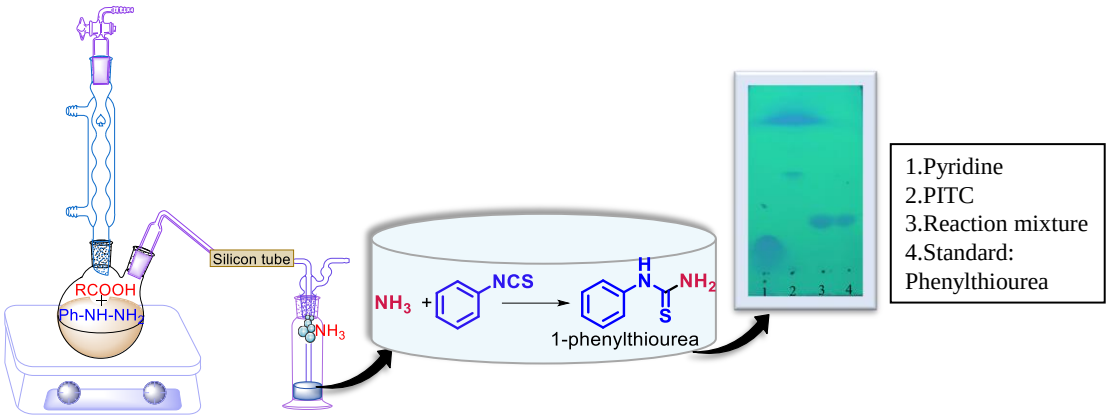


Figure 4.8. Ammonia trap experiment.

4.4.1.4. Outcomes

This is the first unique report where hydrazines have been disclosed as amine surrogates for the direct amidation of carboxylic acids in the presence of $ZnCl_2$. Comparatively, the process offers a clean protocol for amide synthesis in which ammonia was only a by-product. Reaction protocol has scope for further modification towards the environment-friendly process as it tolerates the semi-aqueous reaction medium. Reaction protocol appears robust over the wide variety of substrates; it tolerates many other functionalities, as revealed by the reaction of phenylhydrazines with various carboxylic acid-containing compounds, including amino acids, drugs, and natural products. Using this reaction protocol of amidation, various amide derivatives of beddomeilactone were synthesized via reaction with methoxy, ethoxy, and halogen-substituted phenylhydrazine. Five beddomeilactone amide derivatives were obtained in good yield (>60%) (Figure 4.9).

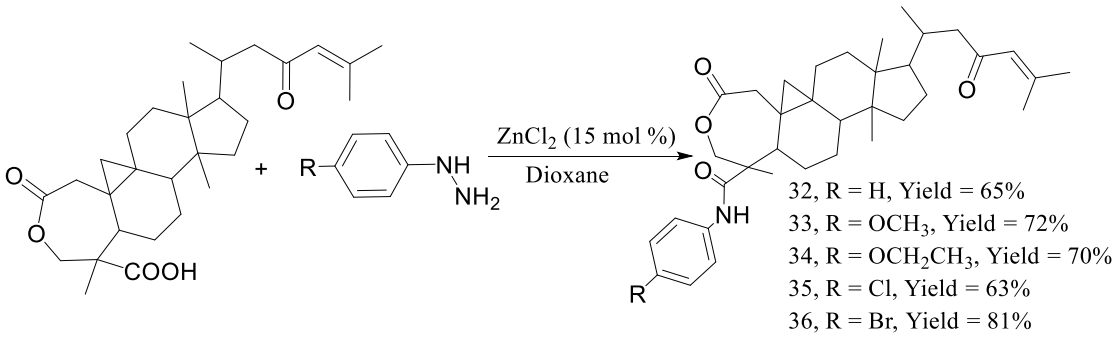


Figure 4.9. Synthesized amide derivatives of beddomeilactone.

The synthesized amide derivatives (compounds **32-36**) were evaluated for cytotoxicity screening using MTT assay against breast cancer cell lines MCF-7, T-47D and MDA-MB-231. The findings showed that significant cytotoxicity was detected in a concentration-dependent manner. The percent viability of breast cancer cells decreased as the compound concentration increased. All the compounds were active against these cell lines and showed IC_{50} in the range of 15-45 μM. Compound **32** showed significant cytotoxicity against all three cell lines and was most cytotoxic against T-47D breast cancer cells (IC_{50} 16 μM). Also, compound **32** was more cytotoxic than beddomeilactone against breast cancer cells.

4.4.2. Series 2: Synthesis of halogenated derivatives

The halogenated derivatives of beddomeilactone were synthesized to introduce functionalization at the double bond in the aliphatic side chain. For the same, the halogenation of beddomeilactone was planned using N-halosuccinamide. Beddomeilactone was first hydrogenated using a palladium catalyst, and then further bromination was done using N-bromosuccinamide to get the brominated product, as shown in Figure 4.10.

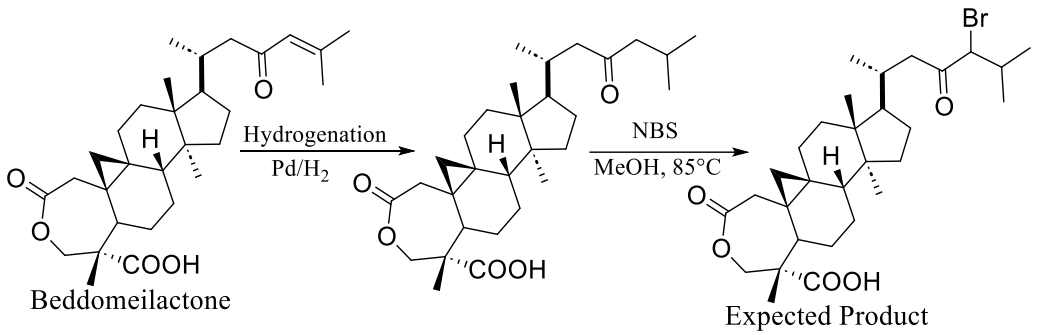


Figure 4.10. Proposed halogenation reaction of beddomeilactone.

After the hydrogenation of beddomeilactone, the obtained reduced double-bond derivative was subjected to a bromination reaction. Surprisingly, unexpected product formation was observed in the reaction, and 30% of the starting material remained

unreacted. The HRMS spectrum of the isolated and purified compound showed a major peak at *m/z* 579.2941 $[M+H]^+$. The reaction resulted in bromination at the aliphatic side chain along with esterification at ring A of beddomeilactone, which can be observed in Figure 4.11 a and b.

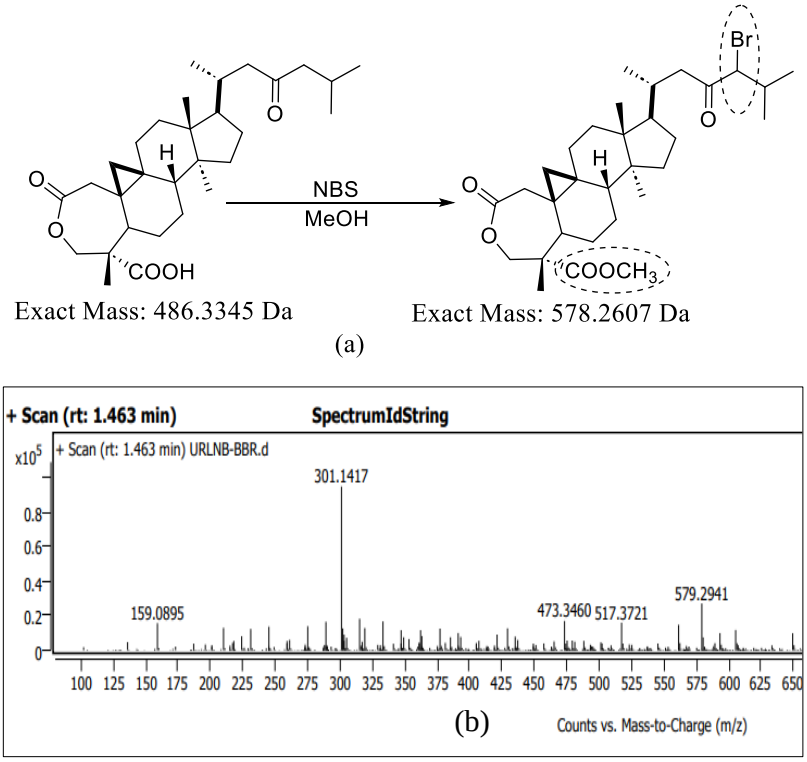


Figure 4.11. (a) Bromination of beddomeilactone (b) HRMS spectrum of the product obtained.

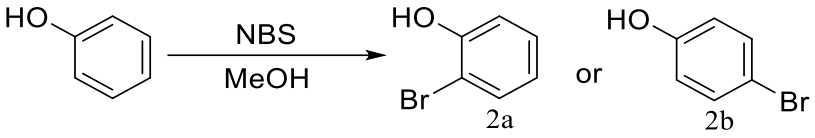
Later, the reaction condition was optimized using various aromatic acids where esterification of the acid group was observed with only a few acids. Also, the ester formation in the product was uncertain, so the reaction was evaluated further for other substrates like phenol and aldehydes.

4.4.2.1. Optimization of reaction condition

To develop a protocol for the efficient halogenation of phenolic compounds with NXS in an aqueous or semi-aqueous medium, the first commercially available NiO (25 wt%) as the catalyst was utilized. The initial success was achieved with a 20% yield producing both ortho- and para-bromophenol in an aqueous medium. The addition of methanol

improved the yield to 40% at a short reaction time and a low temperature (entries 1 and 2, Table 4.2).

Table 4.2. Optimization of the nuclear bromination reaction.



Entry	Catalyst	Reaction conditions	% Conversion	Yield (%)[a]	
				ortho	*para*
1	NiO (commercial)	H_2O, 100 °C, 8 h	20	8	12
2	NiO (commercial)	MeOH: H_2O (8:2),60°C, 4 h	40	19	21
3	Ni-NiO (wt 25%)	H_2O, rt, 4 h	68	8	60
4	Ni-NiO (wt 25%)	MeOH: H_2O (8:2), rt, 1 h	100	10	90
5	Ni-NiO (wt 5%)	MeOH: H_2O (8:2), rt, 1 h	100	15	85
6	Ni-NiO (wt 10%)	MeOH: H_2O (8:2), rt, 1 h	100	8	92
7	Ni-NiO (wt 15%)	MeOH: H_2O (8:2), rt, 1 h	100	8	92

[a]Analyzed by GCMS.

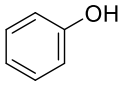
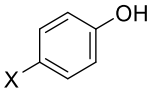
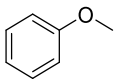
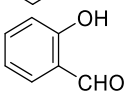
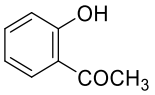
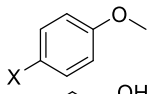
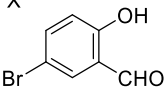
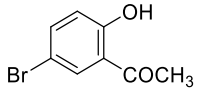
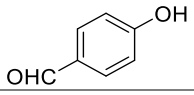
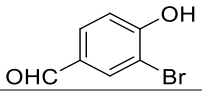
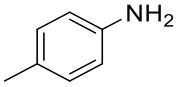
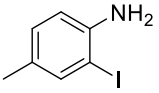
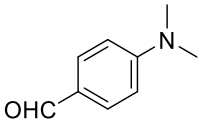
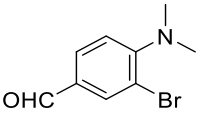
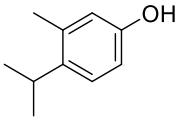
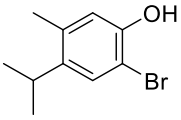
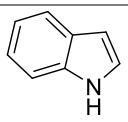
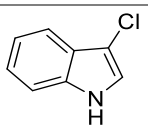
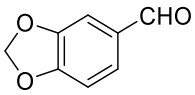
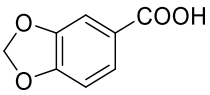
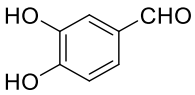
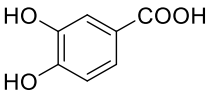
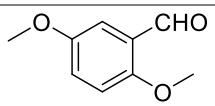
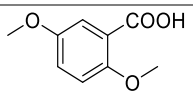
After that, Ni-NiO nanoparticles were used for the catalytic halogenations of phenol. In water, the reaction of phenol and NBS in the presence of Ni-NiO (4 h at room temperature) yielded 60% of para-bromophenol and 8% ortho-bromophenol. A tremendous improvement in the activity and selectivity was observed in methanol: water (8:2) mixture when 90% of p-bromophenol and 10% of o-bromophenol were produced after 1 h reaction at room temperature. Further optimization has shown that 10 wt% of Ni-NiO is the optimum amount to achieve regioselective bromination and the addition of a higher amount of catalyst does not make any significant difference in the product yield and in the reaction time. Finally, the optimized protocol for regioselective halogenation involves the use of Ni-NiO nanoparticles 10 wt% (w.r.t. phenol) in methanol: water (8:2) at room temperature and 1 equivalent of phenol and NBS (Table 4.2).

4.4.2.2. Synthesized products of halogenation and esterification

The regioselective performance of the catalyst under optimized protocol was confirmed over EDG-substituted (-Me, -NH_2) aromatic substrates. In all the cases, the formation of p-bromo-substituted product was observed as the major product under the optimized conditions. To investigate the substrate-specific reactivity of the catalyst, various EWGs-substituted (-OCH_3, -$COCH_3$, -CHO) aromatic/benzene were also tested (Table 4.3). In all the cases, *para*-selectivity was achieved under the optimized conditions (entry 2 in Table 4.3). For example, the reaction of salicylaldehyde with NBS and Ni-NiO led to the formation of a *para*-bromination product with 92% yield (*para* relative to the hydroxy group; entry 2 Table 4.3). It was worth mentioning here that blocking of the para-position resulted in ortho-brominated products. When positions 3 and 4, relative to carbonyl were occupied, nuclear bromination was retarded. Reactions of 3,4-methylene-dioxybenzaldehyde, 3,4-dihydroxybenzaldehyde, and 2,5-dimethoxybenzaldehyde did not produce any halogenated product (entry 9 Table 4.3). Instead, a minor amount of corresponding benzoic acid was recovered along with the starting material. This result can be explained by the effect of substituents, which make the ortho/para position of the aromatic ring less electrophilic.

As a minor amount of benzoic acid was detected as the product in the previous examples (entry 9 in Table 4.3), we investigated the scope of Ni-NiO nanoparticles-mediated oxidation of different carbonyl compounds (Table 4.4). Benzaldehyde was taken as a substrate for the model reaction to establish a suitable protocol for the oxidation reaction. In an aqueous medium, Ni-NiO nanoparticles catalyzed the oxidation to form benzoic acid as the single product. The rapid oxidation of benzaldehyde to benzoic acid can be attributed to the activation of oxygen by the catalyst present in water. Furthermore, it was noteworthy that a radical mechanism cannot be dismissed in this process.

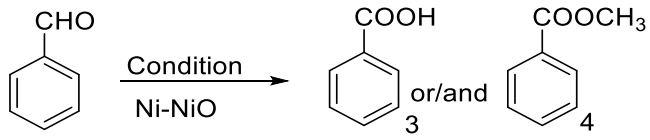
Table 4.3. Halogenation of aromatic compounds.

Entry	Substrate	Major product	Yield
Effect of electron-donating substituents in the aromatic ring			
1			(i) X = Br 96% (ii) X = Cl 91% (iii)X = I 94%
Effect of electron-withdrawing substituents in the aromatic ring			
2.	  	  	(i) X = Br 89% (ii)X = Cl 85% 92% 92%
Blocking of *para*-position of the aromatic ring			
3.			90%
4.			(i) X = I 88% (ii)X = Br 84%
5.			90%
			75%
Selectivity in the five-membered ring			
6.			80
Blocking of both 3,4-positions in the aromatic ring			
7.			28
8.			25
9.			18

[a]Reaction conditions: aromatic phenolic or non-phenolic substrate (1 mmol), NBS (1 equiv.), MeOH: water (8:2), 1 h; [b]Yield of major product.

A control experiment was performed without the addition of the catalyst to get a minor amount of benzoic acid even after 16 h of reaction. The same reaction of benzaldehyde in the methanol: water (8:2) produced methyl benzoate as the major product (85%) and the reaction was completed at a low temperature and reduced reaction time (2 h at 50 ^{o}C). In the absence of the catalyst, a very small amount of benzoic acid was obtained after 16 h of reaction, and the formation of methyl benzoate was not observed under control conditions. This observation clearly indicates that Ni-NiO nanoparticles catalyze the esterification simultaneously, and the reaction does not proceed through the oxidation of the benzaldehyde to acid and further to the ester.

Table 4.4. Oxidation of aromatic benzaldehydes catalyzed by Ni-NiO nanoparticles.

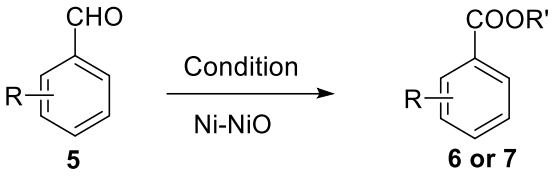


Entry	Solvent	Temperature (^{o}C)	Amount of NBS (eqv)	Catalyst	Time (h)	Major Product	Yield of major product
1.	H_2O	100	0	Ni-NiO	2	Benzoic acid	80[c]
2.	H_2O	100	0	No catalyst	16	Benzoic acid	40[c]
3.	MeOH: H_2O[b]	50	0	Ni-NiO	2	Methyl benzoate	85
4	MeOH: H_2O[b]	50	0	No catalyst	16	Benzoic acid	55[c]
5	MeOH: H_2O[b]	35	0.25	Ni-NiO	1	Methyl benzoate	95
6	MeOH: H_2O[b]	Rt	0.25	Ni-NiO	1	Methyl benzoate	75
7	MeOH	80	0.25	No catalyst	12	Methyl benzoate	50[c]
8	MeOH	80	1	No catalyst	12	Benzoic acid	50[c]

[a]Reaction conditions: aromatic carbonyl compound (1 mmol), NBS (1 equiv.), catalyst Ni-NiO NPs (10 wt%) and solvent. [b]MeOH: H_2O in 8:2 ratio, the reaction didn't complete in given condition and starting material remained unreacted.

Again, the protocol was optimized to investigate the role of NXS in the oxidation process, which performed the reaction in the presence of catalyst and NXS. Methyl benzoate was obtained with a 95% yield and 0.25 equiv of NBS. These results were compared with the reaction of benzaldehyde and NBS without adding the catalyst. The expected product methyl benzoate was obtained in a moderate yield of 75% after 1 h. A catalytic amount (0.25 equiv.) of NBS alone can produce a 50% yield, but only after 12 h of reaction. With 1 equiv. of NBS in methanol: water (8:2) produced benzoic acid in an overall yield of 50%. It has been reported that NBS facilitates esterification reactions under dark and basic conditions. However, our optimized protocol for esterification using Ni-NiO nanoparticles requires a catalytic amount of NBS in semi-aqueous conditions of methanol: water (8:2). Further, the optimized protocol was used to examine the scope of other alcohols (entry 1-6, Table 4.5). Substitution of methanol, with ethanol or allyl alcohol, produced corresponding esters as the major product (entries 2, 3 in Table 4.5). It was observed that when non-alcoholic solvents such as THF, DMF, DCM, dioxane, or acetonitrile were used, the carboxylic acid was formed instead of carboxylic ester (entries 7-10, Table 4.5).

Table 4.5. Oxidative reaction of benzaldehyde in the presence of Ni–NiO nanoparticles



5

6 or 7

7: R' = Alkyl

5a, R = *p*-NO_2
5b, R = *p*-Cl
5c, R = *p*-Br
5d, R = 3-Br,4-OCH_3
5e, R = *p*-F
5f, R = *m*-Cl

6: R' =H
6a, R = *p*-NO_2, R' = H
6b, R = *p*-Cl, R' = H
6c, R = *p*-Br, R' = H
6d, R = *p*-F, R' = H

7a, R = *p*-NO_2, R' = Me
7b, R = *p*-NO_2, R' = Et
7c, R = *p*-NO_2, R' = allyl
7d, R = *p*-Cl, R' = Me
7e, R = *p*-Br, R' = Me
7f, R = 3-Br,4-OCH_3, R' = Me
7g, R = *m*-Cl, R' = Me

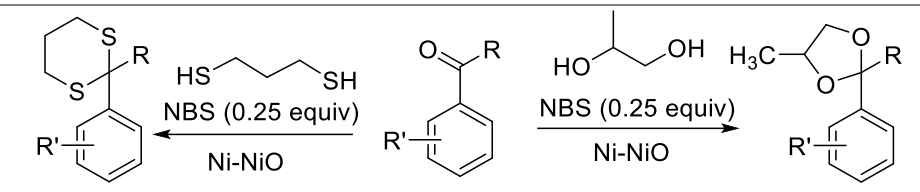
Entry	Substrate	Solvent	Major Product	Yield[b]
1	5a	MeOH: H_2O	7a	92
2	5a	EtOH: H_2O	7b	88
3	5a	Allyl-OH: H_2O	7c	86
4	5b	MeOH: H_2O	7d	90
5	5c	MeOH: H_2O	7e	91
6	5d	MeOH: H_2O	7f	89
7	5a	DMF	6a	92
8	5b	CH_3CN	6b	90
9	5c	CH_2Cl_2	6c	90
10	5e	Dioxane	6d	90
11	5f	MeOH: H_2O	7g	88

[a]Reaction conditions: aromatic carbonyl compound (1 mmol), NBS (1 equiv.), catalyst Ni-NiO NPs (10 wt%) and solvent for 2h; [b]Yield of major product.

Further, the protocol was again tested for ketone substrates. Ni-NiO nanoparticles catalyzed the acetalization, ketalization and thioketalization of ketone in the presence of the catalytic amount of NBS with diol and dithiol. These reactions are important in the protection of the carbonyl group and widely applicable in synthetic chemistry. It is also important to mention here that acetalization and thioacetalization compete with esterification, especially, in the case of benzaldehydes. Hence, reactions in dioxane, such as water or non-alcoholic solvent, offer better yield. The reaction of benzaldehyde, diol, or dithiol in MeOH and H_2O yielded both ester and acetal or thioacetal. Desired products were obtained

in good yield in 1-2 h (Table 4.6). Furthermore, it was noticed that when a high equivalent of NBS was used for the substrate with EDG, nuclear halogenation, as well as oxidation reaction, occurred under the optimized conditions of the catalyst (Figure 4.12).

Table 4.6. Acetalization, ketalization and thioketalization of carbonyl compounds by NBS.



9a, R = R' = H
9b, R = H, R' = 2-Cl
9c, R = H, R' = 3-Cl
9d, R = H, R' = 4-NO_2
9e, R = CH_3, R' = H
9f, R = CH_3, R' = 4-Cl
9g, R = CH_3, R' = 2,6-di-Cl

8a, R = R' = H
8b, R = H, R' = 2-Cl
8c, R = H, R' = 3-Cl
8d, R = CH_3, R' = 2,6-di-Cl
8e, R = H, R' = 4-NO_2
8f, R = H, R' = 4-Cl
8g, R = H, R' = 3-Br
8h, R = H, R' = 4-F
8i, R = CH_3, R' = H
8j, R = CH_3, R' = 4-Cl

10a, R = H, R' = 4-NO_2
10b, R = H, R' = 2-Cl
10c, R = H, R' = 3-Cl
10d, R = H, R' = 4-Cl
10e, R = H, R' = 3-Br
10f, R = H, R' = 4-F
10g, R = CH_3, R' = H
10h, R = CH_3, R' = 4-Cl

Entry	Substrate	Major Product	Yield[b]
1	8a	9a	85
2	8b	9b	78
3	8c	9c	83
4	8d	9d	75
5	8e	10a	75
6	8b	10b	77
7	8c	10c	78
8	8f	10d	83
9	8g	10e	85
10	8h	10f	88
11	8i	10g	90
12	8j	10h	85

[a]Reaction conditions: The aromatic carbonyl compound (1 mmol), NBS (0.25 eqv.), catalyst (10 wt%), diol or dithiol, and solvent for 1 h; [b]Yield of major product.

The treatment of p-N, N´-dimethyl amino benzaldehyde with Ni-NiO nanoparticles and NBS (3 equiv.) in MeOH: H_2O produced a mixture of three products **A**, **B** and **C**. To study the rate and preference of these reactions, initially, p-N, N´-dimethyl

amino benzaldehyde was treated with 1 equiv. NBS and Ni-NiO NPs to produce only mono-brominated product **A** in 95% yield. The addition of an extra 1 equivalent of NBS led to the formation of product **B**. Further addition of the third equivalent of NBS produced methyl ester product **C**. This study clearly indicated that the rate of nuclear halogenation was higher than the oxidation reaction for substrate p-N, N´-dimethyl amino benzaldehyde and the desired product can be isolated by sequentially adding required moles of NBS. These results indicate that an electron-rich aromatic system favours electrophilic substitution reaction.

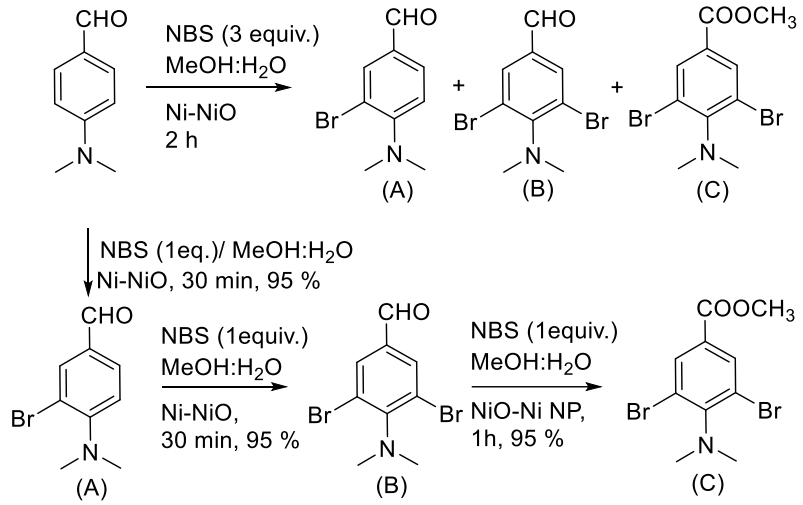


Figure 4.12. Competitive nuclear bromination and oxidative esterification. Reaction conditions: (1 mmol), NBS (1 equiv.), MeOH: water (8:2), 1 h.

4.4.2.3. Plausible reaction mechanism

For the mechanism of nuclear halogenation, it might operate through an electrophilic substitution reaction. The possibility of the free radical mechanism as it underwent smoothly in the presence of free-radical scavengers such as (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) was dismissed. For the mechanism of oxidative esterification, it may proceed through hemiacetal intermediate, which gets oxidized by the catalyst in presence of NBS to the corresponding hemiacetal hypohalite followed by elimination of hydrogen halide to yield an oxidized

product. The esterification of aldehyde through acid as an intermediate can be ruled out as methyl benzoate couldn't be obtained from benzoic acid under the standard optimized conditions [126].

4.4.2.4. Outcomes

This study concluded that aromatic carbonyl compounds with a deactivated ring system favours oxidation via nucleophilic addition *viz.* oxidative esterification, acidification, acetalization, ketalization, thioacetalization and thioketalization, where the order of reactivity was NO_2 > halogen. The catalyst was optimized for regeoselective halogenation and esterification on aromatic carbonyl compounds and phenolic substrates respectively. A remarkable *para*-selective halogenation was achieved for phenolic substrates under semi-aqueous conditions. Interestingly, the deactivated ring system favours oxidation via nucleophilic addition. Among aldehydes and ketones, aldehydes were more reactive compared to ketones towards oxidative esterification.

Using the optimized reaction condition, the brominated beddomeilactone derivative, as described in Figure 4.10, was synthesized and further evaluated for cytotoxicity screening against breast cancer cells. The brominated derivative exhibited IC_{50} of 16.87 μM against T-47D cell lines, 20.25 μM against MCF-7 and 30.63 μM against MDA-MB-231 breast cancer cell lines. Therefore, the result indicated that the brominated derivative was found to be more cytotoxic than the starting material, beddomeilactone.