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# *p*-Toluenesulfonyl Chloride Catalysed Facile Synthesis of *O*-benzyl-L-amino Acids and Their In Vitro Evaluation

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## Abstract

Protection and subsequent deprotection of amino acid functional groups play a key role in regioselective peptide synthesis. For protection, carboxylic acid functional groups are often benzylated using *p*-toluenesulfonic acid catalysed Fischer-Speier esterification reaction. Such reaction involves in situ water formation, which requires subsequent separation by azeotropic distillation for forward shift of equilibrium. To eliminate the need of this corresponding step requiring additional set-up, current study investigated *p*-toluenesulfonyl chloride as a reasonable alternative catalyst for facile benzylation of selected mono- and di- carboxylic amino acids. Literature reports that *p*-toluenesulfonyl chloride not only has a better shelf life but also demonstrates better safety in case of accidental systemic absorption over *p*-toluenesulfonic acid. As the *O*-benzyl-L-amino acids are often retained without deprotection to constitute the pharmaceutical peptide systems, synthesized compounds were investigated for their biocompatibility using in vitro cytotoxicity assays.

**Keywords** Amino acids · Benzylation · Esterification · Peptide synthesis

## Introduction

Organic synthesis requires permutation of reagents and catalysts for clean synthesis of desired products, without formation of undesired products through side reactions. Protecting groups are often used to diminish such side reactions; since these are the molecules that mask and exclude extra functional groups from reacting during synthesis. This is one of the approaches undertaken to achieve regioselective bond formation in organic synthesis. A chemical with the following three fundamental properties can be used as a protecting group in organic synthesis: (i) its association with target functional group should be easy, (ii) the association should remain stable over a broad range of reaction conditions, (iii) its dissociation from synthesized moiety i.e., deprotection should be easy and harmless (Isidro-Llobet et al. 2009).

Peptides often constitute the pharmaceutical delivery systems. Protection and subsequent deprotection of amino acid functional groups play a key role in peptide synthesis. As each amino acid individually contain one or more carboxylic acid and amine functional groups, their planned protection becomes necessary for regioselective peptide synthesis. Carboxylic acid functional groups are protected through their esterification, owing to simplicity of the reaction, product stability as well as inertness during coupling reactions and ease of deprotection at final stage to obtain the desired compound. Hence, amino acid esters form important intermediates for peptide synthesis.

Fischer-Speier esterification reaction is often employed for protecting the carboxylic acid functional group. The reaction (Fig. 1) is used for derivatizing carboxylic acid function to its methyl, ethyl, benzyl etc. esters by refluxing the former with excess of respective alcohol in presence of an acidic catalyst such as hydrochloric acid (HCl), sulfuric acid ($H_2SO_4$) or *p*-toluenesulfonic acid (*p*-TsOH). Due to the better purity of esters formed and ease of catalyst neutralisation during product purification, *p*-TsOH is preferred over HCl and $H_2SO_4$ (Furniss et al. 1996). As esterification employing alcohol and carboxylic acid is reversible,

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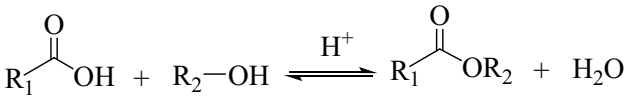


**Fig. 1** Fischer-Speier esterification reaction

an equilibrium exists; which can be rapidly driven forward towards ester formation by azeotropic distillation of water formed in situ. Hence, Fischer–Speier esterification reaction is often carried out in presence of organic solvents like benzene or toluene forming heterogeneous azeotrope with water (Furniss et al. 1996; Wang 2010).

Bolchi et al. investigated the impact of water azeotroping solvents employed for Fischer-Speier esterification reaction on environmental safety and product racemization. The significant observations reported were (i) water azeotroping solvents such as benzene (b.p. 80.1 °C) and carbon tetrachloride (b.p. 76.72 °C) are environmentally hazardous, and (ii) high-boiling, water azeotroping solvents such as toluene (b.p. 110.6 °C) or neat benzyl alcohol (b.p. 205 °C) give scope for product racemization. Although cyclohexane (b.p. 81 °C) was reported as a suitable solvent, azeotropic distillation of water was still necessary as corresponding step requiring additional set-up (Bolchi et al. 2015, 2017, 2018).

*p*-TsOH is a white, crystalline, water soluble solid available in its anhydrous and monohydrate forms. Although both forms assume deliquescent nature on long atmospheric exposures, as compared to the anhydrous form (m.p. 38 °C) its monohydrate form (m.p. 103–106 °C) is more stable. However, due to its hygroscopic nature the monohydrate compound is often observed to absorb moisture during storage and turn pink in colour. Hence, shelf life of *p*-TsOH is highly influenced and limited by the storage conditions practiced (SDS 2018a). Alternatively, due to its water insolubility and anhydrous nature, *p*-TsCl demonstrates better stability and subsequently better shelf life over *p*-TsOH (SDS 2018b). Moreover, based on results of toxicity studies conducted in the rodent models, literature indicate that in case of accidental systemic absorption *p*-toluenesulfonyl chloride (*p*-TsCl) (oral-rabbit $LD_{50}$: 4680 mg/kg) is safer than *p*-TsOH (oral-rat $LD_{50}$: 2480 mg/kg) (Chemicalbook 2019a, b).

Hence current study (Fig. 2) investigated application of *p*-TsCl as an alternative catalyst for benzylation of selected mono- and di- carboxylic amino acids. Use of *p*-TsCl not only eliminated the need of tedious azeotropic distillation, but also presented an alternative catalyst over *p*-TsOH with better shelf life and safety (in case of accidental systemic absorption). Furthermore, biocompatibility of the formed products was investigated using in vitro cytotoxicity assays; since *O*-benzyl-L-amino acids are often retained without deprotection to constitute the pharmaceutical peptide systems.

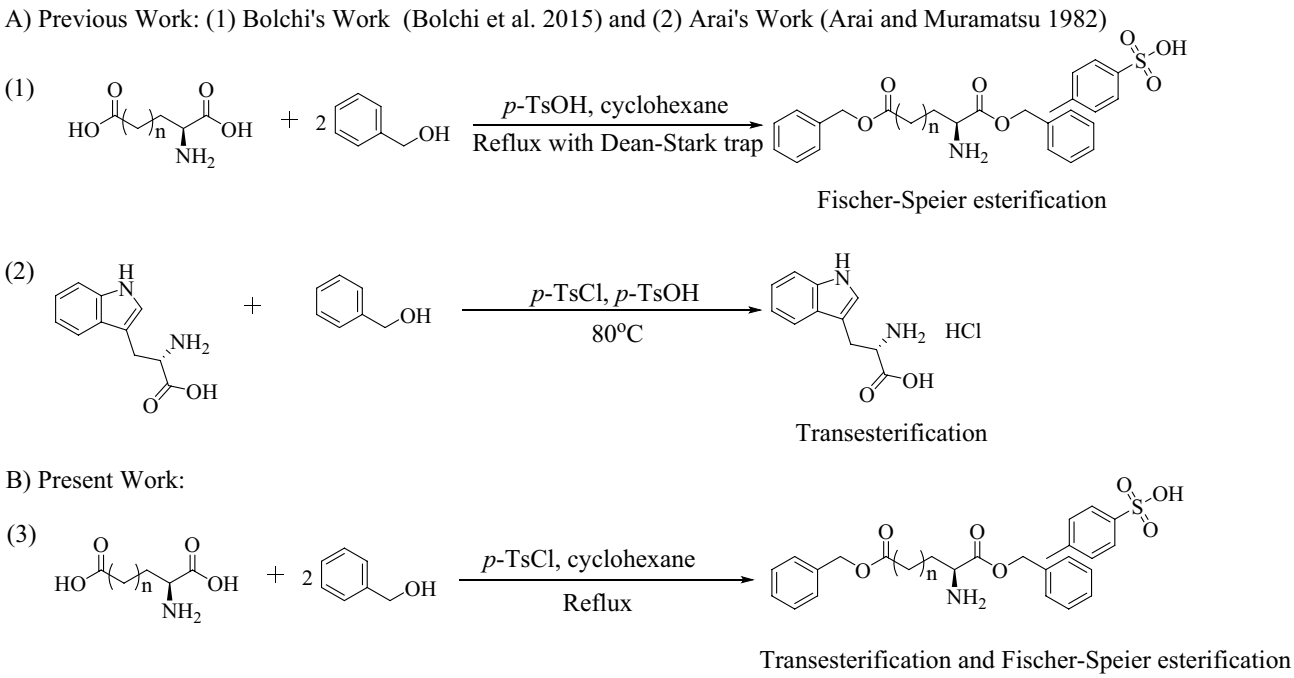



**Fig. 2** Summary of difference between previous work and present work

## Experimental

### Materials and Methods

#### Chemistry

Extra pure grade L-glutamic acid, L-aspartic acid and L-phenylalanine were purchased from S. D. Fine Chemical Limited (India). The analytical grade solvents and *p*-TsCl were procured from Research-Lab Fine Chem Industries (India). Reagent grade benzyl alcohol and cyclohexane were ordered from Thermo Fisher Scientific India Pvt. Ltd. (India) and Spectrochem Pvt. Ltd. (India), respectively. Progress of the reaction was monitored by thin-layer chromatography (TLC) using silica gel 60 $F_{254}$ supported on aluminium plate (Merck). Fourier-Transform infrared (FTIR) spectra of the synthesized molecules were recorded using Perkin Elmer Spectra RXI FTIR spectrometer. Mass spectra of synthesized molecules were recorded using Shimadzu LCMS-8040 triple quadrupole LC–MS/MS spectrometer having Electrospray Ionisation (ESI) source. Nuclear Magnetic Resonance (NMR) spectra of synthesized molecules were recorded using Bruker NMR spectrometer. Mettler Toledo DSC 1 STAR$^e$ Thermogravimetric Analyser was used to perform thermal analysis of all the synthesized compounds.

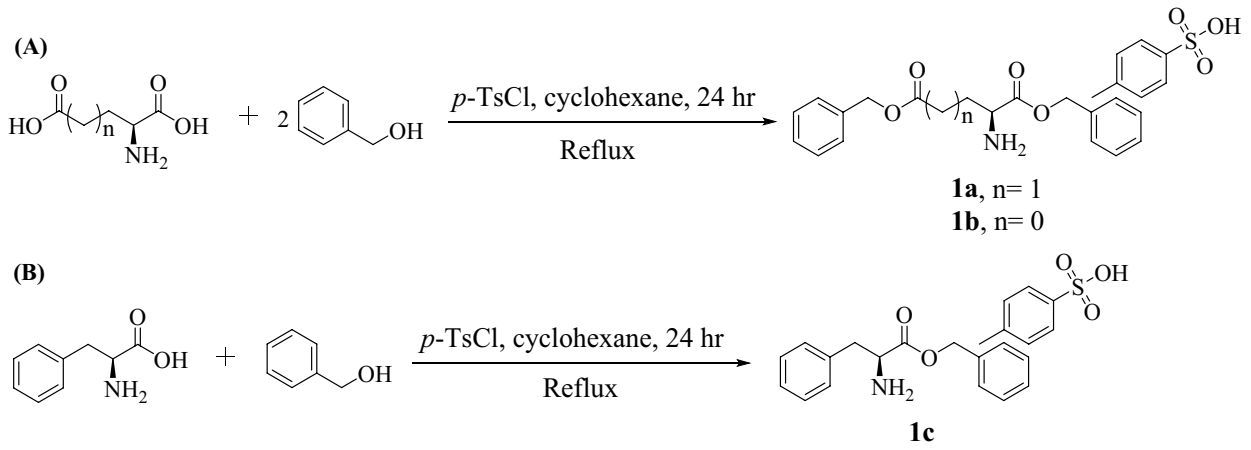
#### Synthesis of 1a–1c

General procedure for synthesis of **1a**–**1c**: *p*-TsCl catalysed benzyl esterification of mono- and di- carboxylic acid is described in Scheme 1. For L-phenylalanine, since it contains monocarboxylic group, half equivalents of benzyl alcohol and *p*-TsCl were taken than those used for dicarboxylic amino acids L-aspartic acid and L-glutamic acid. Reaction mixture contained 1 gm (6.8 mmol) L-glutamic acid, 1.56 gm (8.2 mmol) *p*-TsCl, 4 ml (38.6 mmol) benzyl alcohol and 20 ml (excess) cyclohexane. The reaction mixture was refluxed overnight under continuous stirring and progress of reaction was monitored through TLC. On completion, the reaction mixture was cooled to room temperature and ethyl acetate was added till complete precipitation of products **1a**–**1c**. The precipitate obtained was filtered, washed with cold ethyl acetate and dried to yield benzyl-protected *p*-toluenesulfonate salts of respective amino acids.

L-Glutamic acid dibenzyl ester *p*-toluenesulfonate (**1a**): obtained as a white solid (2.48 g, 81%) m.p. 130.31 °C, $R_f$ (TLC, methanol/ dichloromethane 1:9) = 0.83, IR (KBr, $cm^{-1}$): 3471.40 (N–H stretching), 1743.30, 1731.58 (C=O stretching), 1599.39 (N–H bending). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.41 (s, 3H), 7.51 (d, $J$ = 8.1 Hz, 2H), 7.48–7.33 (m, 10H), 7.13 (d, $J$ = 7.6 Hz, 2H), 5.32–5.18 (m, 2H), 5.11 (s, 2H), 4.18 (t, $J$ = 6.5 Hz, 1H), 2.61 (q, $J$ = 8.4, 7.7 Hz, 2H), 2.30 (s, 3H), 2.12 (tq, $J$ = 14.6, 8.1, 7.3 Hz, 2H). MS (ESI) m/z calcd for $C_{19}H_{22}NO_4^+$ $[M+H]^+$ 328.15; found 328.25; MS (ESI) m/z calcd for $C_7H_7SO_3^-$ $[M–H]^-$ 171.01, found 170.40.

L-Aspartic acid dibenzyl ester *p*-toluenesulfonate (**1b**): obtained as a white solid (3.17 g, 87%) m.p. 159.95 °C, $R_f$ (TLC, methanol/ dichloromethane 1:9) = 0.87, IR (KBr, $cm^{-1}$): 3455.46 (N–H stretching), 1758.20, 1734.26 (C = O stretching), 1593.17 (N–H bending). $^1$H NMR (400 MHz, DMSO-d6) δ 8.45 (s, 2H), 7.46 (d, J = 8.1 Hz, 2H), 7.40–7.27 (m, J = 3.9, 3.3 Hz, 10H), 7.08 (d, J = 7.8 Hz, 2H), 5.16 (s, 2H), 5.07 (s, 2H), 4.46 (t, J = 5.4 Hz, 1H), 2.99 (qd, J = 17.5, 5.4 Hz, 2H), 2.25 (s, 3H). MS (ESI) m/z calcd for $C_{18}H_{20}NO_4^+$ $[M+H]^+$ 314.14, found 314.10; MS (ESI) m/z calcd for $C_7H_7SO_3^-$ $[M–H]^-$ 171.01, found 170.90.

L-Phenylalanine benzyl ester *p*-toluenesulfonate (**1c**): obtained as a white solid (1.89 g, 73%) m.p. 164.91 °C, $R_f$




**Scheme 1** Synthesis of *O*-benzyl amino acids, **a** dibenzylation of L-glutamic acid (**1a**) and L-aspartic acid (**1b**) and **b** benzylation of L-phenylalanine (**1c**)

(TLC, methanol/dichloromethane 1:9) = 0.285, IR (KBr, $cm^{-1}$): 3450.98 (N–H stretching), 1741.29 (C = O stretching), 1602.79 (N–H bending). $^1$H NMR (400 MHz, DMSO-d6) δ 8.44 (s, 3H), 7.72–6.78 (m, 14H), 5.18–5.05 (m, 2H), 4.35 (t, J = 6.1 Hz, 1H), 3.22–2.95 (m, 2H), 2.26 (s, 3H). MS (ESI) m/z calcd for $C_{16}H_{18}NO_2^+$ $[M+H]^+$ 256.13, found 256.10; MS (ESI) m/z calcd for $C_7H_7SO_3^-$ $[M–H]^-$ 171.01, found 171.00.

### Cytotoxicity Study

Intrinsic cytotoxicity of the synthesized compounds was determined by MTT assay using A549 (adenocarcinoma) and T3T (fibroblast) cells. MTT assay was performed as described earlier with minor modifications (Juvale et al. 2013). Briefly, cells were trypsinised at 90% confluence and 5000 cells/well were seeded into 96-well plates. The plates were then incubated for 24 h at 37 °C and 5% $CO_2$. After the incubation, test compounds were added at final concentration of 25 µM in triplicates and the plates were further incubated for 72 h. MTT dye (5 mg/ml) was then added to each well and incubated for next 2 h. The supernatant was removed and 100 µL of DMSO was added to each well to dissolve formazan crystals formed. Optical density of formazan was measured at 570 nm using Molecular Devices ID3 Multi-mode Plate Reader. % cell viability was calculated by considering untreated cells as negative control and gefitinib treated cells as positive control.

## Results and Discussion

### Synthesis

In the current study *p*-TsCl was used to synthesize *p*-toluenesulfonates of selected mono- and di- carboxylic amino acid esters without employing azeotropic distillation of water. In the synthesis, benzyl alcohol was used as a benzylating agent, cyclohexane was used as a low-boiling solvent and *p*-TsCl was used as a catalyst for transesterification. Since similar reaction using *p*-TsOH catalyst was also reported by Bolchi et al. (Bolchi et al. 2015), we compared practical yields of L-glutamic acid esterification obtained by both the methods, keeping equivalent ratios of all the components same. % yield of former was 86.69% whereas that of latter was 80.80%, indicating acceptable yields with *p*-TsCl without employing azeotropic distillation.

In contrast to the work reported by Arai et al., present work reports a reaction that requires only *p*-TsCl as catalyst and involves a low-boiling solvent. Arai et al. has reported benzylation of tryptophan by transesterification that requires addition of nearly same equivalents of *p*-TsCl as well as *p*-TsOH. Addition of *p*-TsCl to benzyl alcohol forms benzyl *p*-toluenesulfonate. Since *p*-toluenesulfonate is a good leaving group, it is then replaced by carboxylate to form the benzyl ester of tryptophan. *p*-TsOH was primarily used to improve solubility of amino acid in benzyl alcohol in absence of any solvent, and also to maintain acidic environment for preventing side-reaction between *p*-TsCl and amine function of amino acid (Arai and Muramatsu 1982).

We propose that in presence of *p*-TsCl catalyst, the benzylation reaction assumes mechanism which is a combination of transesterification and Fischer-Speier esterification

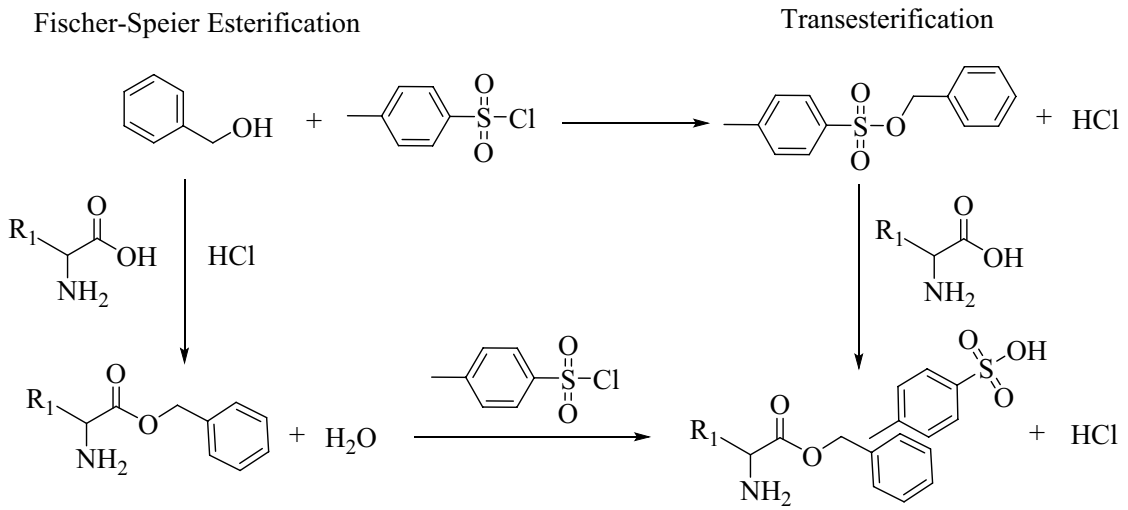



**Fig. 3** Plausible mechanism of benzyl esterification in presence of *p*-TsCl catalyst

(Fig. 3). Benzyl alcohol and *p*-TsCl form the intermediate benzyl *p*-toluenesulfonate. Carboxylate group of amino acid replaces *p*-toluenesulfonate to form *p*-toluenesulfonate salt of benzyl ester of amino acid as desired product and HCl as a by-product. The HCl thus formed in situ also behaves as a catalyst and facilitates the Fischer–Speier esterification reaction in conventionally known way. As water molecule formed is consumed by *p*-TsCl to form *p*-TsOH, azeotropic distillation is no longer required. When reaction temperature is allowed to drop to the room temperature, the *p*-toluenesulfonate benzyl ester of amino acid precipitates as a stable salt on addition of ethyl acetate. Hence, for the aforementioned reaction *p*-TsCl catalyst kick starts benzylation of amino acid via transesterification and the HCl formed in situ as a by-product further catalyses the reaction similar to Fischer-Speier esterification.

Also, the overall pH of reaction mixture is maintained low not only due to the acidic catalyst *p*-TsCl but also due to the by-products like HCl. This ensures absence of any basic component in the reaction. Maintenance of such acidic conditions prevent reaction between the chloride catalyst and amino group, eliminating scope of other by-product formation (Arai and Muramatsu 1982).

FTIR spectra recorded for synthesized compounds showed significant shift in carbonyl- peaks from $\nu_{max} \sim 1650\ cm^{-1}$ to $\nu_{max} \sim 1750\ cm^{-1}$, indicating conversion of carboxylic acids to carboxylic esters, respectively. During mass analysis, *p*-toluenesulfonates of amino acid benzyl esters ionized strongly being salts, producing signals in positive as well as negative ionisation modes. For all the synthesized molecules, the base peak in positive ionisation mode $[M+H]^+$ varied depending upon the mass of amino acid benzyl ester. In negative ionisation mode, the precursor m/z $[M–H]^-$ was found to be ~ 171. Anhydrous *p*-TsOH demonstrates $[M–H]^-$ value of 171.01 as its molar mass is 172.2 g/mol. Hence, $[M+H]^+$ values confirmed *O*-benzylation of respective amino acids, while $[M–H]^-$ values confirmed their isolation in the form of stable *p*-toluenesulfonate salts. Moreover, presence of singlet peak for methyl protons of *p*-toluenesulfonate group is observed in NMR spectra of all the synthesized compounds at chemical shift values between 2.25 and 2.30 ppm. This observation is consistent with the results obtained in reported works (Bolchi et al. 2015, 2017). Hence, the analytical data confirms formation of stable *p*-toluenesulfonate salts of *O*-benzyl amino acids in presence of *p*-TsCl catalyst and supports the proposed reaction mechanism.

All the synthesized molecules were further subjected to differential scanning calorimetry (DSC) studies to confirm the enantiomeric excess of the synthesized compounds. Figure 4 gives the representative DSC thermogram for compound **1a.** Compound **1a** exhibited a single endothermic peak at 130.31 °C, confirming enantiomeric excess of the analysed compound. DSC thermograms of compounds **1b** and **1c** (included in Online Resource Figure S10 and S15) also showed presence of single endothermic peaks at 159.95 °C and 164.91 °C respectively, confirming enantiomeric excess of synthesized compounds.

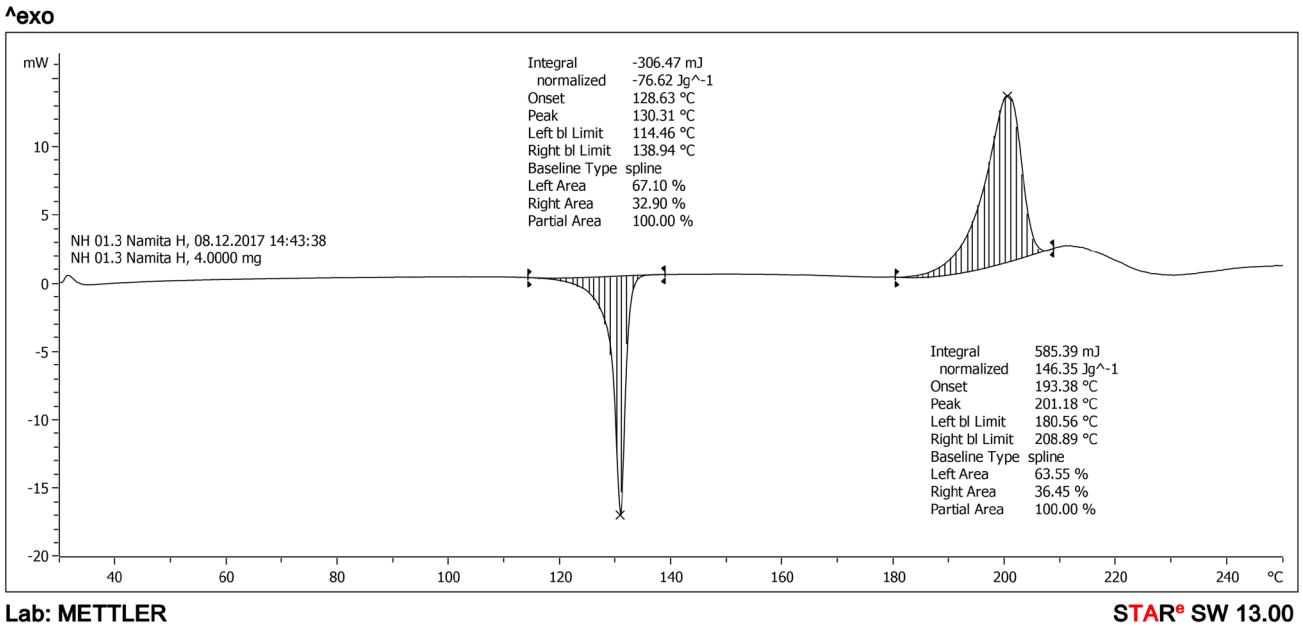



**Fig. 4** DSC thermogram of compound **1a**

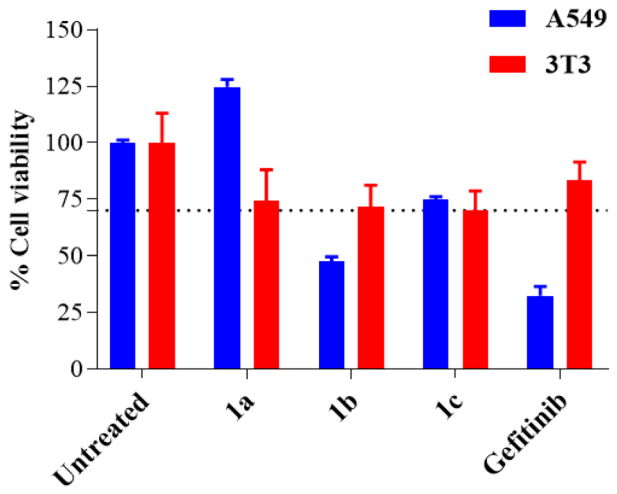



**Fig. 5** Results of in vitro cytotoxicity assays of **1a**–**1c** in 3T3 and A549 cells

### In Vitro Assay

Protected amino acids may be retained as such without deprotection to form a part of peptide systems designed for drug delivery (Hegde et al. 2019; Duan et al. 2011). Hence, we felt the need to confirm biocompatibility of *p*-toluenesulfonates of benzyl-protected amino acids using in vitro cytotoxicity assays. Synthesized compounds were investigated at 25 µM concentrations in A549 (adenocarcinoma) and 3T3 (fibroblast) cell lines using untreated cells as negative control and gefitinib treated cells as positive control. The results are compiled in Fig. 5.

In 3T3 (fibroblast) cells, all the three compounds as well as gefitinib demonstrated more than 70% cell viability. Gefitinib does not show considerable cytotoxicity in 3T3 cells as these does not overexpress the EGFR receptors. Similarly, the synthesized compounds also did not demonstrate significant cytotoxicity towards fibroblast cells. In A549 (human lung carcinoma) cells, compound **1a** and **1c** showed more than 70% cell viability indicating lack of significant cytotoxicity for adenocarcinoma cells as well. However, compound **1b** demonstrated less than 50% cell viability in A549 cells indicating certain associated anticancer activity. This information can be helpful while selecting and utilizing these molecules as components of peptide systems designed for drug delivery.

## Conclusion

Benzylation of carboxyl group of the amino acids is one of the most commonly used approaches for adding regioselectivity to the reactions involved in peptide synthesis. Current study reports *p*-TsCl mediated facile benzyl esterification of selected amino acids. We propose that in presence of *p*-TsCl, benzylation of amino acid assumes a mechanism which is combination of transesterification and Fischer-Speier esterification, and does not require elimination of in situ formed water by azeotropic distillation. The *p*-TsCl catalysed esterification resulted into *p*-toluenesulfonates of amino acid benzyl esters with > 70% yields. Furthermore, in vitro studies of the synthesized compounds revealed a possible anticancer activity of **1b** (L-aspartic acid dibenzyl ester *p*-toluenesulfonate) in A549 (adenocarcinoma) cell line, which needs further investigation. At 25 µM concentrations, all the three compounds were demonstrated to be safe in 3T3 (fibroblast) cell line, confirming their biocompatibility.

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### Compliance with Ethical Standards

**Conflict of interest** Authors declare that they have no conflict of interest.

**Ethical Approval** No experiments involving human participants or animals were performed for this article.

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