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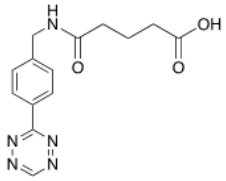
An efficient metal catalyst free approach to synthesize 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid Berhanu Zewde , Olufolasade Atoyebi, Dharmaraj Raghavan 	Leave this area blank for abstract info.

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ABSTRACT

Despite the wide use of 1,2,4,5- tetrazines in biomaterials and materials science, currently there does not exist synthetic method(s) that can yield significant amount of 1,2,4,5- tetrazines without the use of potentially toxic metal catalysts. Here, we report a less energy intensive and more efficient metal catalyst free approach for the synthesis of an asymmetric tetrazine. A range of operating parameters such as extraction pH and temperature were regulated to achieve a practical yield nearly 1.5 times greater than the yields reported in the literature for similar synthetic procedures.

1. Introduction

In recent years, there has been a significant interest in the use of 1,2,4,5-tetrazines as coupling agents for a range of biological applications.[1,2] Tetrazine has been used in bioorthogonal reactions for intracellular small molecule imaging, genetically targeted protein tagging, post synthetic DNA labeling, and nanoparticle-based clinical diagnostics.[3-7] In particular, the ability to combine molecular imaging with the bioorthogonal chemistry of tetrazine has significantly enhanced the utility of the technique for in vitro and in vivo imaging under a variety of conditions. Bioorthogonal reactions have also found utility in protein engineering[8], immune assay development[9] and cell surface modification[10]. Tetrazine compounds have also found utility in materials science,[11] coordination chemistry,[12] and explosives research[13].

Generally the synthesis of 1,2,4,5-tetrazines is a two step process involving the addition of hydrazine to an aromatic nitrile followed by oxidation of the 1,2-dihydrotetrazine intermediate.[14] Commonly, a nitrile compound with a carboxylic acid or amine functionality is chosen as the precursor for synthesis so as to promote coupling of tetrazine with dyes or biomacromolecules. Also, the synthesized tetrazine must be asymmetric to prevent unintended crosslinking. The yields of the tetrazines in the absence of catalyst are typically 10–17%[15-16].

The work by Yang *et al*. demonstrated that by the addition of 0.05 equivalents of nickel or zinc triflate catalysts, the tetrazine yield can be significantly enhanced i.e. 4 fold over catalyst free reactions.[14] However, the use of significant amount of nickel triflate catalyst can limit the broad use of 1,2,4,5- tetrazines for applications in biological coupling due to concerns of metal toxicity. Also yields of the 1,2,4,5-tetrazine can be compromised because the reaction mixture has to be exposed to high temperature and/or pH conditions during workup. Alge et al. addressed the ill effects of metal catalysts by exploring the effects of concentration of lewis acid on the synthesis of 1,2,4,5-tetrazines.[16] By eliminating sulfur and conducting the reaction with hydrazine and formamidine acetate in the presence of 0.005 equivalents of nickel triflate, Alge et al obtained the tetrazine in yields as high as 75%. Although Alge et al. reported high yield with reduced metal catalyst requirement over what has previously been used,[14] there is a need for synthesizing the tetrazines in large yield without metal catalyst so as to eliminate the potential impact of residual metal catalyst on biological activity of the compound.

Since biomaterials applications require large amounts of hydrogel, we began to explore methods to refine and improve the yield of 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid (compound 2), a precursor to hydrogel synthesis. In our study, the Alge et al. [16] procedure was slightly modified by conducting the reaction in the absence of metal catalyst and using milder conditions to recover optimal amount of the final product. First we synthesized 5-(4-(cyano)benzylamino)-5-oxopentanoic acid (compound 1) from 4-(aminomethyl)benzonitrile hydrochloride. This was followed by synthesis of compound 2 by combining compound 1 with formamidine acetate salt, elemental sulfur, and anhydrous hydrazine. Extraction pH and temperature were found to influence the yield of asymmetric tetrazine. Practical yields as high as 27% were achieved.

The stability of compound 2 in various media can be equally important for the design and development of peptides for use in laboratory and the clinical studies.[17-19] There have been reporting of

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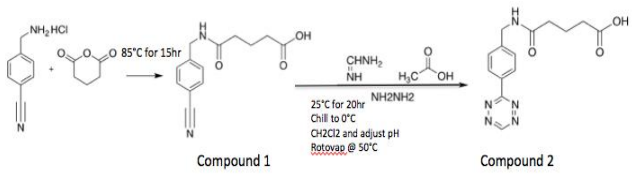
stability studies of tetrazine derivatives in phosphate buffer saline (PBS) or fetal bovine serum (FBS).[15, 20] However, there has been no detailed investigation about tetrazine stability in lysogeny broth (LB) media and no comparative stability data for all the media available in the literature. As part of this study, we investigated thermal stability and stability of compound 2 in PBS buffer, LB broth, and FBS.

2. Experimental

All chemicals for the experiment were purchased from Sigma Aldrich, and used as received except glacial acetic acid from Fisher Scientific, and silica gel (70 and 230 mesh size) from Alfa Aesar.

2,1 *Synthesis of 5-(4-(cyano)benzylamino)-5-oxopentanoic acid and 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid*

We adopted the Alge et al. method (as outlined in Scheme 1) to synthesize compound 1 with slight modification[16]. To 4-(aminomethyl)benzonitrile hydrochloride, glutaric anhydride, trimethylamine, acetonitrile was added and the mixture was refluxed, acidified and extracted with ethyl acetate to eventually yield white solid compound (~85%).



Scheme 1 Reaction scheme for synthesis of 5-(4-(cyano) benzylamino)-5-oxopentanoic acid (compound 1) and 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid (compound 2)

Scheme 1 also describes the procedure outlined to synthesize compound 2. Compound 1, formamidine acetate salt, elemental sulfur, and anhydrous hydrazine were combined with sodium nitrite in glacial acetic acid. The pink solution formed was cooled and dichloromethane was added, followed by base addition until the formation of a separate layer. Then the organic layer was recovered, dried, washed with brine, dried over $MgSO_4$ and washed with dilute acid to obtain compound which was subsequently purified by column chromatography and characterized by FTIR, LC/MS and NMR.

The ^{1}H NMR of compound 1 was found to be consistent with literature reporting.[21] Observation of NMR peak in compound 2 at δ =10.58 (s, 1H) is a strong indication of tetrazine group formation in step 2 of target compound synthesis.

To confirm the successful synthesis of compounds 1 and 2, FTIR results were collected and significant similarities in the spectra of both compounds were observed. Observation of carboxylic acid group, the amide group, and the N-H bending group from the amide band in the FTIR spectra of the compound 2 is in line with the structure of 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid. Additionally, we notice in the spectrum of compound 2, a weak peak at 1665 cm^{-1}, assigned to C=N of the imine, which is not observed in the spectrum of compound 1, confirming the successful synthesis of the target compound. Also, we notice a medium sharp peak at 2231.89 cm^{-1}, representing the C≡N group, in the spectrum of the compound 1 which is not noticed in the spectrum of compound 2.[24] These observations strongly suggest that the target compound was indeed synthesized.

NMR, FTIR results were corroborated with LC-MS results which further supported the successful synthesis of compound 2.

2.2 Optimization Study

Compound 2 was recovered from reaction mixture using methylene chloride as the extractant and a basic solution for pH adjustment. As the pH was increased, the target compound partitions from the aqueous phase into the organic phase. By performing multiple extractions, more of the target compound was recovered from the aqueous phase into the organic phase. The concentration and purification of the compound after every batch extraction was followed by scanning the solution from 250 nm to 750 nm using a HP 8453 diode array UV-Vis spectrophotometer (**Figure 1b**).

2.3 Stability Measurements of *5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid*

To establish that compound 2 was prone to degradation at elevated temperatures, we subjected the reaction mixture to elevated rotovap temperature and UV-Vis absorbance of the diluted rotovap solution was compared against absorbance of standard solution.

Biological media stability study was conducted by preparing solution of compound 2 in DMSO and PBS, FBS, or LB broth and incubating at 37 ^{o}C. The degradation of compound 2 in the media was followed by monitoring the absorbance of the solution at 530 nm over 24h and comparing the absorbance against a standard solution.

3. Results and Discussion

3.1 Optimization Study to Maximize the Yield of 5-(4-(1,2,4,5-tertazin-3-yl)benzylamino)-5-oxopentanoic acid

The yellow slurry obtained during step 2 of compound 2 synthesis was dissolved with glacial acetic acid. Addition of sodium nitrite yielded a bright pink compound and significant amount of gas liberation. However the removal of glacial acetic acid from reaction by rota vapor required the use of relatively high temperature. To establish whether the use of relatively high rotovap temperature indeed influences the yield of the target compound (because of degradation of the compound 2), a systematic study was conducted by subjecting a known amount of reaction mixture to varying rotovap temperature and diluting the recovered dried sample with methylene chloride to a known volume followed by UV-Vis assay. **Figure 1a** shows the absorbance plot of samples (that has been diluted) as a function of rotovap temperature. As expected, when the solution was rotovaped at temperatures greater than room temperature (50 ^{o}C) the absorbance of recovered sample was significantly higher than the solution rotovaped at room temperature implying the evaporation of the solvent at temperature around 50 ^{o}C yields more concentrated compound 2. Further increase in rotovap temperature (to $\geq$ 60 ^{o}C) so as to concentrate the compound 2 resulted in a decrease in the absorbance of the sample. In fact, the absorbance of the solution decreased substantially upon subjecting the solution to significantly higher rotovap temperature. Our results suggest that rotovap temperatures $\geq$ 60 ^{o}C results in the degradation of the target compound which are consistent with observations reported by Bird et al. where a slow decomposition of compound was noticed at 60 ^{o}C.[25] By conducting the temperature stability study for 7 days at 90 ^{o}C, Bird et al. noticed that most of the compound 2 decomposed. Therefore, in our subsequent optimization studies, we subjected our reaction mixture to rotovap temperatures of ~50 ^{0}C.

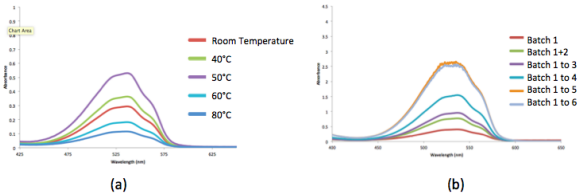


Figure 1 Absorption scan of 5-(4-(1,2,4,5-tetrazin-3-yl)benzyl amino)-5-oxopentanoic acid as a function of (a) rotovap temperature and (b) extraction batch used in concentrating reaction mixture.

Optimization study to recover the target compound from the reaction mixture included the use of methylene chloride as the extractant along with NaOH. NaOH addition is expected to neutralize the acetic acid in the reaction mixture. To improve the yield of the target compound, we chilled the reaction mixture and dichloromethane was added to the reaction mixture so as to partition the target compound into the organic phase. Despite the addition of lesser polar dichloromethane to polar water, we observed only one phase mixture suggesting that acetic acid in the reaction mixture possibly serves as an emulsifier in improving the miscibility of the organic phase with the aqueous phase. As the pH was increased,

separation of the organic phase from the aqueous phase was noticed because the glacial acetic acid reacted with NaOH to form salt. Compound 2 which was originally present in the homogeneous organic-aqueous mixture starts partitioning into the organic phase (pink coloration), upon NaOH addition. By performing multiple extractions with methylene chloride, the compound was recovered successfully from the aqueous phase into the organic phase. The first batch extract was concentrated and pH was found to be 2.4. We repeated the protocol of addition of methylene chloride, and NaOH, and concentration on the concentrate to recover compound 2. **Figure 1b** shows the absorbance plot of organic phase concentrate after multiple batch extractions. The absorbance of the compound 2 increased after each subsequent batch extraction with marginal difference in absorbance between 5th and 6th batch suggesting that the optimum concentration of compound 2 was reached. Practical yields of 27% were achieved via our optimization study, which is 1.5 times greater than the yields reported in the literature for similar synthetic procedure.[21] The improved yield could be largely attributed to the use of optimum rotovap temperature to minimize the degradation of compound during concentration and use of solvent exchange protocol as well as pH condition to efficiently concentrate and purify the target compound from the reaction mixture.

3.2 Stability of 5-(4-(1,2,4,5-tertazin-3-yl)benzylamino)-5-oxopentanoic acid in Biological Media

The stability of compound 2 in biological media was followed by performing UV-Vis scan of the incubated solution from 250 nm to 750 nm. Compound 2 showed strong absorbance at 530 nm which was assigned to the n→π* transition.[27] Our maximum absorbance was compared to the absorbance of the standard curve so as to calculate the percent of compound decomposed at varying incubation period.[26,27] **Table 1** shows the % of compound 2 decomposed in (a) FBS, (b) PBS and (c) LB media at various periods of incubation at 37 ^{o}C. In PBS solution, the compound showed minimal degradation over 24 h with an average decomposition of 23.25 %. In our study, we also noticed during the initial 6 h of incubation, an 8.76 % decrease in the original absorbance. Our results are in general agreement with Zhongqiu et al. [20] observation where a small 3.3 % decrease in the absorbance was noticed in the initial 6 h of incubation, for a similar tetrazine compound incubated at 20 °C. On the other hand, in FBS solution, the compound showed significant degradation. After 24 h incubation, the compound decomposed by an average 76.61 %. Karver et al. [15] noticed 60 % of the tetrazine degraded after 10 h of incubation at 37 °C; whereas, our study showed an average of 51.84 % of the tetrazine degraded after 18 h of incubation at 37 °C. Our study also noticed that the tetrazine was most stable in LB media with only 16.62 % of the compound decomposed over the 24 h incubation at 37 °C. These results suggest that stability of the target compound in biological media follow the following trend i.e. FBS < PBS < LB.

Table 1 Summary of % of 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid degradation in biological media

	Average percent degradation after:			
Medium	3h	6h	18h	24h
PBS	7.71	8.76	16.6	23.25
FBS	41.97	Not available	51.84	76.61
LB	12.29	11.87	13.64	16.62

4.0 Conclusion

Here, we report a high yielding synthetic procedure for obtaining carboxylic acid functionalized asymmetric tetrazine. A range of operating parameters such as pH, solvent extraction, and temperature were found to influence the recovery of asymmetric tetrazine. Improved recovery has been achieved by successfully working up the reaction mixture with NaOH and performing multiple solvent extractions with CH_2Cl_2 followed by concentrating the extracts at 50 ^{o}C using rotovap. Our workup procedure is less energy intensive and produces 1.5 times yields over that reported in the literature for similar synthetic procedure. In general, tetrazine, showed a varying degree of stability in nutrient medium over 24 h with FBS being the least stable, and LB media being the most stable at 37^{o}C. This new methodology provides an avenue to synthesize compound 2 in larger yields for potential use in biomaterials.

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Highlights:

- Optimized the yield of target compound using suitable workup procedures
- Used environmentally benign research methodology to recover the target compound
- Monitored the biological and thermal stability of tetrazine by UV-Vis Spectroscopy