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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

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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.

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 [7]. Commonly, a nitrile compound with a carboxylic acid or an 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% [14,15].

The work by Yang et al. demonstrated that by the addition of 0.05 eq. of nickel or zinc triflate catalysts, the tetrazine yield can be significantly enhanced i.e. 4-fold over catalyst free reactions [7]. 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 [15]. By eliminating sulfur and conducting the reaction with hydrazine and formamidine acetate in the presence of 0.005 eq. 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 [7], 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. [15]. procedure was slightly modified by conducting the reaction in the absence of metal catalyst and using milder

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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 [16–18]. There have been reporting of stability studies of tetrazine derivatives in phosphate buffer saline (PBS) or fetal bovine serum (FBS) [14,19]. 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.

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.

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 [15]. To 4-(aminomethyl)benzonitrile hydrochloride, glutaric anhydride, trimethylamine, acetonitrile was added under nitrogen and the mixture was refluxed, acidified and extracted with ethyl acetate to eventually yield a white solid compound (~85%).

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 ^{1}H NMR.

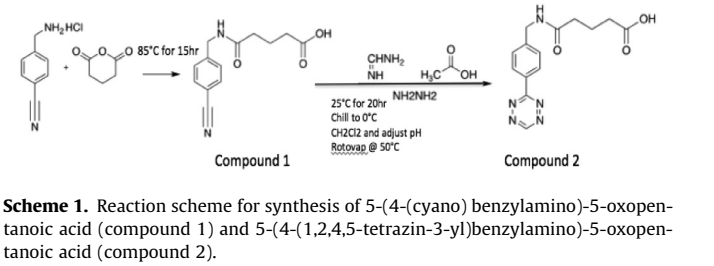
The ^{1}H NMR of compound 1 was found to be consistent with literature reporting [20]. Observation of NMR peak in compound 2 at δ = 10.58 (s, 1H) is a strong indication of tetrazine group formation in step 2 of target compound synthesis.

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 the carboxylic acid group, the amide group [21], and the N=H bending group from the amide band [22] 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 [23]. 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.

Optimization study

Compound 2 was recovered from the 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 (Fig. 1b).



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).

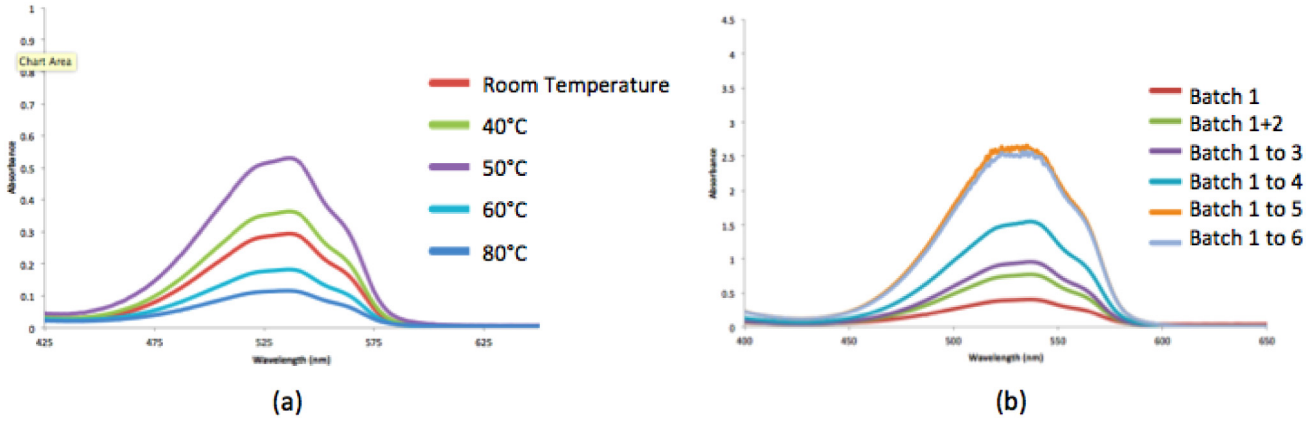


Fig. 1. Absorption scan of 5-(4-(1,2,4,5-tetrazin-3-yl)benzyl amino)-5-oxopentanoic acid as a function of (a) rotovap temperature and (b) extraction batch used in concentrating reaction mixture.