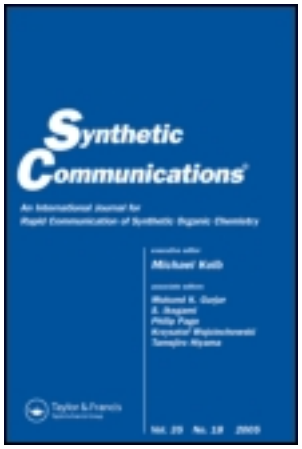

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SYNTHESIS AND CHARACTERIZATION OF METABOLITES AND POTENTIAL IMPURITIES OF BALSALAZIDE DISODIUM, AN ANTI-INFLAMMATORY DRUG

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Balsalazide disodium (Colazide®) is an oral prodrug of mesalamine (5-aminosalicylic acid) and possesses anti-inflammatory properties. During the process development for balsalazide disodium, we observed eight impurities, namely des-β-alanine balsalazide, balsalazide β-alanine, balsalazide 3-isomer, decarboxy balsalazide, bis-azo salicylic acid, biphenyl-azo salicylic acid, bis-azo diacid, and bis-azo triacid. The present work describes the synthesis and characterization of these impurities.

Keywords: 5-Aminosalicylic acid; balsalazide; diazo coupling; impurities; synthesis

INTRODUCTION

Mesalamine, also known as mesalazine or 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug, used to treat inflammation of the digestive tract ulcerative colitis and mild to moderate Crohn's disease.[1–3] Balsalazide is the generic name of 5-[(1E)-[4-[[(2-carboxyethyl) amino]carbonyl]phenyl]azo] 2-hydroxybenzoic acid (**1**).

It is an oral prodrug of 5-ASA, having an inert carrier molecule, *N*-(4-aminobenzoyl)β-alanine (4-ABBA).[4] After oral administration, balsalazide splits into mesalamine and *N*-(4-aminobenzoyl)β-alanine via azo-reduction by the colonic microflora.[5] Mesalamine acts directly on the colon to reduce the local inflammation of the colonic mucosa, which is responsible for the symptoms of ulcerative colitis.[6–8] Balsalazide appeared to be an effective treatment option when compared to salazopyrin. The clinical use and dosage of salazopyrin are limited by adverse reactions,[9–11] several of which are attributed to the absorption of the carrier metabolite sulfapyridine.[12]

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Balsalazide is a therapeutic agent for ulcerative colitis. It is used as disodium dihydrate salt and has molecular formula $C_{17}H_{13}N_3O_6Na_2 \cdot 2H_2O$ and molecular weight 437.32 amu. The commonly used dose of balsalazide is 6.75 g per day, which is equivalent to 2.3 g of mesalamine.[13]

The presence of impurities in an active pharmaceutical ingredient (API) can have a significant impact on the quality and safety of the drug products. Therefore, it is necessary to study the impurity profile of the API to be used in the manufacturing of a drug product. International Conference on Harmonization (ICH) guidelines recommends identification and characterization of all impurities that are present in an API at a level of $\geq$0.05% (based on daily dose).[14] In this context, we have undertaken a comprehensive study to synthesize and characterize the impurities in balsalazide API. These impurities are 5-[[4-[carboxy]phenyl]azo]-2-hydroxybenzoic acid (des-β-alanine balsalazide, **2**); 5-[4-[2-(2-carboxyethyl carbamoyl)ethyl carbamoyl] phenyl azo]-2-hydroxy-benzoic acid (balsalazide β-alanine, **3**); 3-[[4-[[(2-carboxyethyl)amino]carbonyl]phenyl]-azo]-2-hydroxybenzoic acid (balsalazide 3-isomer, **4**); 3-[4-(4-hydroxy phenylazo)benzoylamino]-propionic acid (decarboxy balsalazide, **5**); 2,4-bis-[[4-[(2-carboxyethyl)amino]carbonyl]-phenyl]azo salicylic acid (bis-azo salicylic acid, **6**); 5-[2-[4′,5-bis[(2-carboxyethyl)carbamoyl]-biphenyl-2-yl]diazenyl]-2-hydroxybenzoic acid (biphenyl-azo salicylic acid, **7**); 3-[4-[5-[4-(2-carboxy ethyl carbamoyl)phenylazo]-2-hydroxy phenylazo]benzoyl amino]propionic acid (bis-azo diacid, **8**); and 2,4-bis[[4[[(2-carboxyethyl)amino]carbonyl]phenyl]azo]-3-[4-[[(2-carboxyethyl)-amino]carbonyl]phenyl]phenol (bis-azo triacid, **9**). In addition to these, we have also synthesized and characterized two metabolites, namely 3-(4-acetylamino benzoylamino)-propionic acid (*N*-acetyl-4-ABBA, **10**) and 5-acetylamino 2-hydroxybenzoic acid (5-acetamino-SA, **11**).

Balsalazide disodium was first disclosed in US Patent 4,412,992 (Nov. 1, 1983), assigned to Biorex Laboratories Limited, England, claiming product ''balsalazide'' and process of its preparation. Optimization of this diazonium salt–based process is reported in the literature,[15–19] but to best of our knowledge a detail impurity profile study is not yet cited anywhere, except for four metabolites.[20]

RESULTS AND DISCUSSION

Balsalazide **1** has been synthesized as per Fig. 1.[21] β-Alanine was acylated with 4-nitrobenzoyl chloride **12** in aqueous alkaline solution to give *N*-(4-nitrobenzoyl)-β-alanine **13**.

Hydrogenolysis of **13** with Pd/C in methanol results in the compound *N*-(4-aminobenzoyl)-β-alanine **14**. Diazotization of *N*-(4-aminobenzoyl)-β-alanine **14** with sodium nitrite in aqueous hydrochloric acid yields the diazonium salt **15**, which was coupled with salicylic acid **16** in alkaline medium to give the title product, balsalazide **1**.

Des-β-alanine balsalazide **2**, a carryover impurity, is a result of the presence of 4-nitrobenzoic acid **17** in intermediate **13** of the balsalazide process. This impurity may also form as a result of the amide hydrolysis of balsalazide during the process. Des-β-alanine balsalazide was prepared starting with 4-nitrobenzoic acid **17** according to Fig. 2. Catalytic hydrogenation of **17** with Pd/C in methanol yielded **18**, which was diazotized with sodium nitrite in aqueous hydrochloride and coupled with sodium salicylate to give the desired compound **2** in excellent yield. The electrospray

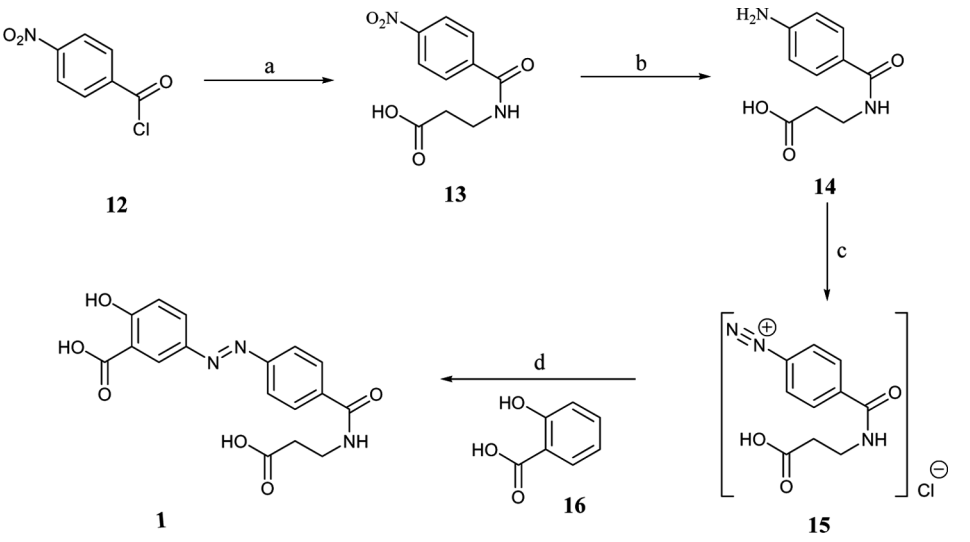

Figure 1. Synthesis of balsalazide **1**. Reagents and conditions: (a) water, NaOH, β-alanine, 0–5 °C, 30 min, 85–90%; (b) methanol, Pd/C, H_2, rt, 2–3 h, 98%; (c) water, conc. HCl, 0–5 °C, aq. $NaNO_2$ solution, 0–5 °C, 1 h, 100%; (d) water, $NaOH/Na_2CO_3$, salicylic acid, 0–5 °C; and then addition of diazonium salt **15** solution, 1 h, 95%.

ionization (ESI) mass spectrum of **2** displayed peaks at m/z 285.2 $[(M - H)^+]$ in negative ion mode and as sodium ion adduct at m/z 307.0 $[(M - H) + Na]$. In positive ion mode, this compound appeared at m/z 287.2 $[(M + H)^+]$ in the mass spectrum. In ^{1}H NMR spectrum of compound **2**, corresponding peaks of β-alanine at δ 2.61 ppm and δ 3.56 ppm were absent. Similarly, in the ^{13}C NMR spectrum, peaks at δ 34.6 ppm and δ 36.6 ppm due to β-alanine moiety were also not observed. The assigned structure for compound **2** is clearly confirmed the spectral data.

Like des-β-alanine balsalazide **2**, balsalazide β-alanine **3** originates from *N*-(4-nitrobenzoyl)di-β-alanine **20**, which may present in intermediate **13** and carry through the synthetic process to give this impurity in the final product.

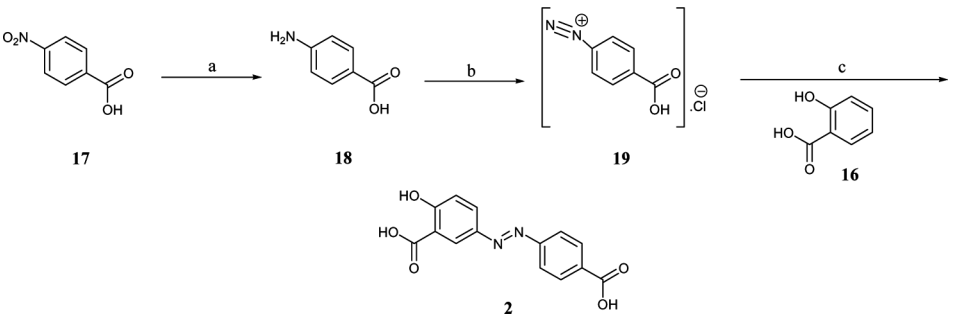

Figure 2. Synthesis of compound **2.** Reagents and conditions: (a) methanol, Pd/C, H_2, rt, 2–3 h, 98%; (b) water, conc. HCl, 0–5 °C then aq. $NaNO_2$ solution, 0–5 °C, 1 h, ~100%; (c) water, $NaOH/Na_2CO_3$, salicylic acid, 0–5 °C, then addition of diazonium salt **19** solution, 1 h, ~100%.

Balsalazide β-alanine **3** was prepared by hydrogenating *N*-(4-nitrobenzoyl)di-β-alanine **20** with Pd/C in methanol to give the corresponding amine **21**, which on diazotization followed by coupling with salicylic acid in an alkaline medium resulted in the desired compound **3** (Fig. 3). The ESI mass spectrum of **3** displayed peaks at m/z 427.2 [(M – H)$^-$] and as sodium ion adduct at m/z 449.0 [(M – H)$^-$ + Na] in –ve ion mode; in +ve ion mode, this impurity appeared at m/z 429.2 [(M + H)$^+$]. The ^{1}H and ^{13}C NMR spectra of compound **3** also support the assigned structure for **3** (di-β-alanine analog of balsalazide).

Balsalazide 3-isomer **4** is a positional isomer of balsalazide **1** and can form as a result of the coupling of diazonium salt **15** to the 3-position of salicylic acid **16** instead of the 5-position (Fig. 4).

N-(4-Aminobenzoyl)-β-alanine **14** as hydrochloride salt was diazotized with aqueous sodium nitrite solution at 0–5 °C and further treated with sodium salicylate in water/isopropyl alcohol mixture at 0–5 °C to yield compound **4** as a by-product along with **1**. The ESI mass spectrum of compound **4** is showing equal mass value as that of balsalazide. The ^{1}H NMR of **4** exhibits three *doublet of doublets* at δ 6.61 ppm (dd, $J = 7.7$ Hz, 1H), 7.57 ppm (dd, $J = 8.0$ Hz and 1.7 Hz, 1H), and 7.87 ppm (dd, $J = 8.0$ Hz and 1.7 Hz, 1H) corresponding to three hydrogens at the 4-, 5-, and 6-positions of the aromatic ring of salicylic acid. ^{13}C NMR also confirmed the assigned structure of compound **4**.

Decarboxy balsalazide **5** was detected in **1** as a degradation product. Decarboxylation of balsalazide results in this impurity. We have prepared this impurity from 4-hydroxybenzoic acid **23,** wherein *N*-(4-aminobenzoyl) β-alanine **14** was diazotized with aqueous sodium nitrite solution/hydrochloric acid at 0–5 °C and added to aqueous alkaline solution of **23** to obtain compound **5** (Fig. 5). The ESI mass spectrum of compound **5** displayed peak at m/z 312.1 [(M – H)$^-$] in –ve ion mode, which is 44 mass units (amu) less than balsalazide (m/z 356.2). In +ve ion mode, its base peak appeared at m/z 314.0 [(MH)$^+$], and the peak corresponded

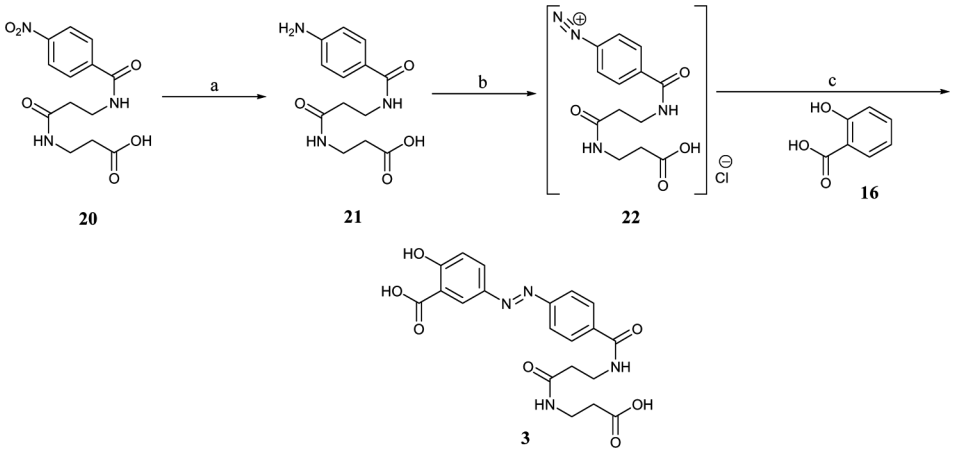

Figure 3. Synthesis of compound **3**. Reagents and conditions: (a) methanol, Pd/C, H_2, rt, 2–3 h, 98%; (b) water, conc. HCl, 0–5 °C aq. $NaNO_2$ solution, 0–5 °C, 1 h, 100%; (c) water, NaOH /Na_2CO_3, salicylic acid, 0–5 °C, then addition of diazonium salt **22** solution, 1 h, 82%.

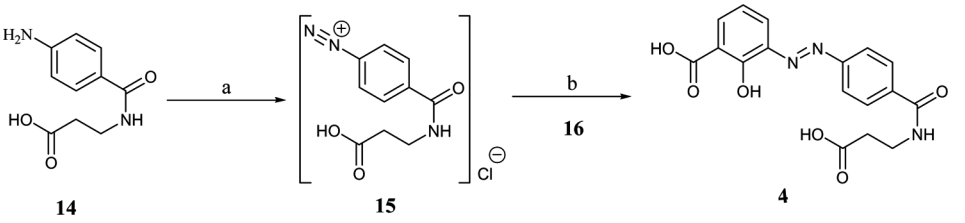

Figure 4. Synthesis of compound **4.** Reagents and condition: (a) water, conc. HCl, 0–5 °C aq. $NaNO_2$ solution, 0–5 °C, 1 h, ~100%; (b) water, $NaOH/Na_2CO_3$, salicylic acid, 0–5 °C, then addition of diazonium salt **15** solution, 1 h, ~7%.

to the sodium ion adduct appeared at m/z 336.0 [(MH) + Na]. The ^{1}H NMR spectrum of this compound shows signals at δ 6.97 (d, $J = 8.8$ Hz, 2H), 7.84 (d, $J = 8.8$ Hz, 2H), 7.88 (d, $J = 8.8$ Hz, 2H), and 8.04 (d, $J = 8.8$ Hz, 2H); these values indicate that both aromatic rings are *para*-substituted. In addition, ^{13}CNMR and DEPT (distortionless enhancement by polarization transfer) spectra also support the assigned structure for the compound, 116.1(CH), 121.9(CH), 125.2(CH), and 128.4(CH).

Bis-azo salicylic acid **6** forms as a result of the coupling of diazo intermediate **15** with balsalazide **1** on the *ortho* position with respect to the phenolic –OH group during its preparation and carries through the process to give this impurity in balsalazide. The synthetic sequence for the preparation of this impurity is depicted in Fig. 6. Using half mole of salicylic acid in diazo coupling reaction yielded this impurity, but because of the number of side reactions, formation of **6** was less. The ESI mass spectrum of impurity **6** displayed a peak at m/z 577.2 [$(MH)^+$], 221 mass unit (amu) more than balsalazide (m/z 356.2), indicating attachment of diazo intermediate **15** to balsalazide. The signals in ^{1}H NMR spectrum of this impurity at δ 7.90 (2d, $J = 8.8$ Hz, 4H) and 8.01 (2d, $J = 8.8$ Hz, 4H) represent two diazo intermediate moieties, the remaining two aromatic peaks at δ 8.15 (d, $J = 2.5$ Hz, 1H) and 8.51 (d, $J = 2.5$ Hz, 1H), due to the benzene ring of salicylic acid. The smaller coupling constant value ($J = 2.5$ Hz) indicates the *meta*-coupling in the benzene ring, which authenticates the assigned structure for compound **6**.

Base-promoted free radical coupling (Gomberg–Bachmann reaction)[22–24] between the aryl diazonium salt **15** and balsalazide leads to the formation of biphenyl-azo salicylic acid **7**. The synthetic sequence to prepare this impurity is

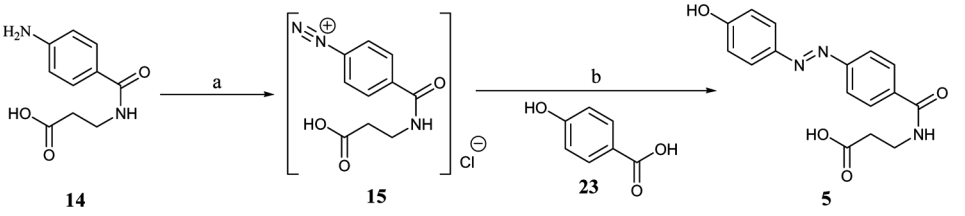

Figure 5. Synthesis of compound **5.** Reagents and conditions: (a) water, conc. HCl, 0–5 °C aq. $NaNO_2$ solution, 0–5 °C, 1 h, 100%; (b) water, $NaOH/Na_2CO_3$, salicylic acid, 0–5 °C, then addition of solution of diazonium salt **15**, 1 h, ~91%.

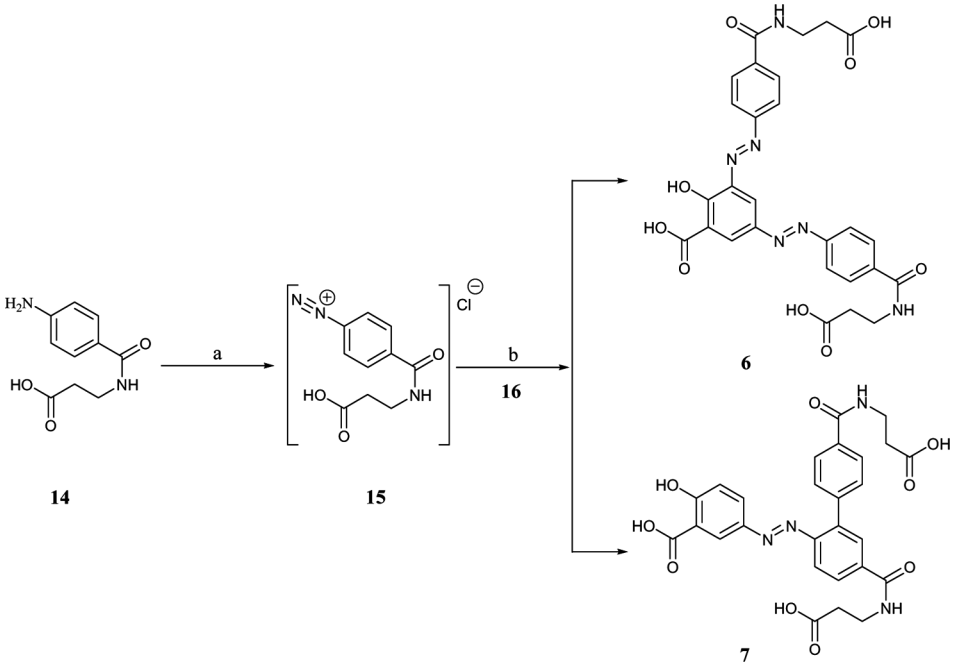

Figure 6. Synthesis of compounds **6** and **7.** Reagents and conditions: (a) water, conc. HCl, 0–5 °C, aq. $NaNO_2$ solution, 0–5 °C, 1 h, ~100%; (b) water, $NaOH/Na_2CO_3$, 0.5 mol eq. salicylic acid, 0–5 °C, then addition of solution of **15**, 1 h, 3–7%.

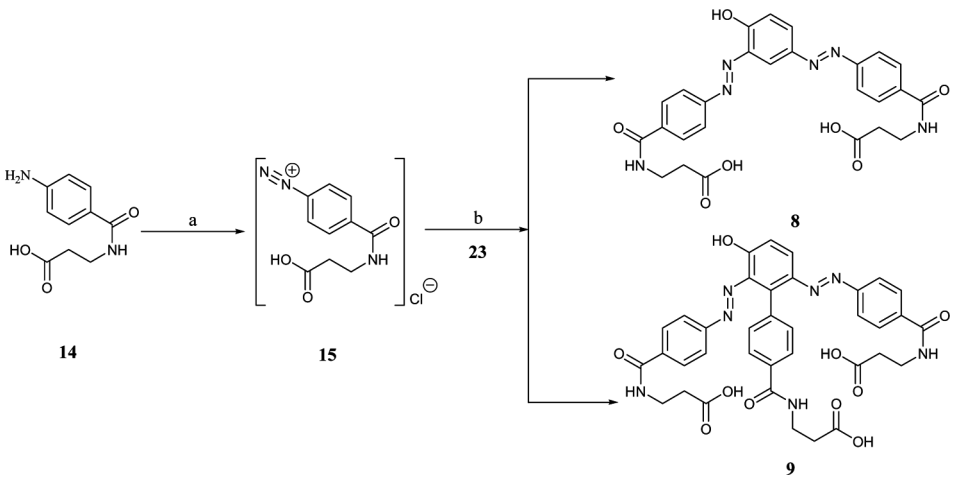

Figure 7. Synthesis of compounds **8** and **9**. Reagents and conditions: (a) water, conc. HCl, 0–5 °C, aq. NaOH solution, 0–5 °C, 1 h, ~100%; (b) water, $NaOH/Na_2CO_3$, 0.5 (for **8**)/0.25 (for **9**) mol eq. 4-hydroxy benzoic acid **23**, 0–5 °C, solution of **15**, 1 h, **8** (~32%) and **9** (~36%).

illustrated in Fig. 6. Using half mole of salicylic acid in the reaction yielded this impurity. The formation of **7** was very less as there were various side reactions. ESI mass spectrum of impurity **7** displayed peak at m/z 549.2 [$(MH)^+$], 28 mass unit (amu) less than bis-azo salicylic acid **6,** indicating diazo group detachment from bis-azo salicylic acid **6**.^{1}H NMR data for compound **6** are 6.70 (d, $J = 8.8$ Hz, 1H), 7.58 (d, $J = 8.5$ Hz, 2H), 7.60 (dd, $J = 8.8$ Hz and 2.5 Hz, 1H), 7.70 (d, $J =$ 8.8 Hz, 1H), 7.93 (d, $J = 8.5$ Hz, 2H), 7.97 (dd, $J = 8.8$ Hz and 1.4 Hz, 1H), 8.05 (d, $J = 1.4$ Hz, 1H), 8.17 (d, $J = 2.5$ Hz, 1H).

Bis-azo diacid **8** originates because of the decarboxylation of compound **6** and contaminates the product. Bis-azo triacid **9** originates because of Gomberg–Bachmann-type coupling of **15** and **8** (Fig. 7). The compound **8** was prepared by reacting diazonium salt **15** with alkaline solution of *para*-hydroxybenzoic acid **23** (0.5 mol equivalent), whereas the compound **9** was obtained by reacting **15** with alkaline solution of 0.25 mol equivalent of **23.**

CONCLUSION

To have a thorough understanding of impurity formation pathways of the anti-inflammatory drug balsalazide disodium, it is essential to have detailed information about the various possible impurities, metabolites, and their synthetic routes. In view of the regulatory importance of the impurities in the API, a detailed study on various impurities in balsalazide was conducted. Different process-related impurities, degradation products, and metabolites in balsalazide API were identified, synthesized, and characterized by using various spectroscopic techniques like low-resolution mass spectroscopy (LCMS), mass,^{1}H NMR, ^{13}C NMR, and infrared (IR).

EXPERIMENTAL

^{1}H NMR, ^{13}C NMR, and DEPT spectral data were performed on a Bruker-Avance 300-MHz spectrometer in dimethylsulfoxide (DMSO-d_6). The chemical shift values were reported on the δ scale in parts per million (ppm), downfield from tetramethylsilane (TMS) as an internal standard. IR spectra were recorded in the solid state as KBr dispersion using a Perkin-Elmer Spectrum One Fourier transform (FT)–IR spectrophotometer. Mass spectrum was recorded using a Perkin-Elmer PE SCIEX-API 2000, equipped with ESI source used online with a HPLC system after the ultraviolet (UV) detector. The solvents and reagents were used without further purification.

5-[[4-Carboxy]phenyl]azo]-2-hydroxybenzoic Acid (Des-β-alanine Balsalazide, 2)

Concentrated hydrochloric acid (30 mL, ~36% w/w) was added to a suspension of 4-aminobenzoic acid (30 g, 0.22 mol) in demineralized (DM) water (360 mL) at 20–25 °C, and thereafter the resultant solution was cooled to 2 to −2 °C. To this solution, aqueous solution of sodium nitrite (15.86 g, 0.23 mol) in DM water (60 mL) was added at 2 to −2 °C. The obtained diazotized solution was added to a precooled alkaline solution of salicylic acid (36.26 g, 0.26 mol) in DM water (630 mL) containing

sodium hydroxide (17.52 g) and sodium carbonate (46.42 g) at 2 to −2 °C, maintaining pH ≥ 8. After completion of reaction, the reaction mass was heated to 60–65 °C, acidified, and stirred for 3 h, maintaining the same temperature. Thereafter, the resulting slurry was cooled to 40 °C, filtered, washed with DM water, and dried to give compound **2** as brown crystalline solid (62.6 g, ~100% yield). IR (KBr, cm^{-1}): 3425 (O−H), 3072 (Ar-CH), 1686 (C=O), 1602 and 1587 (C=C ring), 1457 (aliphatic CH), 1208 (C−N), 1077 (C−O), 799 and 776 (Ar-H, out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 6.96 (d, $J = 8.8$ Hz, 1H), 7.90 (d, $J = 8.5$ Hz, 2H), 7.98 (dd, $J = 8.8$ Hz and 2.5 Hz, 1H), 8.11 (d, $J = 8.5$ Hz, 2H), 8.34 (d, $J = 2.5$ Hz, 1H), 13.20 (brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 116.6 (C), 118.2(CH), 122.1 (CH), 126.5 (CH), 128.0 (CH), 130.6 (CH), 131.9 (C), 143.5 (C), 154.6 (C), 166.8 (C), 166.9 (C), 171.1 (C). MS m/z (ESI): 285.2 [$(M - H)^-$], 307.0 [$(M - H)^- + Na$].

5-[4-[2-(2-Carboxyethyl Carbamoyl)ethyl Carbamoyl]phenyl Azo]-2-hydroxybenzoic Acid (Balsalazide β-alanine, 3)

Concentrated hydrochloric acid (6 mL, ~36% w/w) was added to a suspension of *N*-(4-aminobenzoyl)di-β-alanine (6 g, 0.02 mol) in DM water (72 mL) at 20–25 °C, and the resultant solution was cooled to 2 to −2 °C. To this solution, an aqueous solution of sodium nitrite (1.4 g, 0.02 mol) in DM water (6 mL) was added at 2 to −2 °C. Thereafter, the resulting diazotized solution was added to a precooled solution of salicylic acid (3.2 g, 0.02 mol) in 120 mL DM water containing sodium hydroxide (1.55 g) and sodium carbonate (4.15 g) at 2 to −2 °C, maintaining pH ≥ 8. After completion of the reaction, the resultant mass was heated to 60–65 °C and acidified. The resulting suspension was cooled to 30–35 °C, filtered, washed with DM water, and dried to obtain the desired product **3** (7.7 g, ~82% yield). IR (KBr, cm^{-1}): 3375 (O-H), 3019 (Ar-CH), 1675 (C=O), 1600 and 1577 (C=C ring), 1455 (aliphatic CH), 1212(C-N), 1067 (C-O), 795 and 770 (Ar-H, out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.40 (m, 4H, 2CH_2), 3.26 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 6.77 (d, $J = 8.8$ Hz, 1H), 7.83 (d, $J = 8.8$ Hz, 1H), 7.85 (d, $J = 8.5$ Hz, 2H), 7.97 (d, $J = 8.5$ Hz, 2H), 8.02 (t, $J = 5.5$ Hz, 1H), 8.28 (d, $J = 2.5$ Hz, 1H), 8.62 (t, $J = 5.5$ Hz, 1H), 12.21(brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 34.8 (CH_2), 35.6 (CH_2), 36.2 (CH_2), 37.1 (CH_2), 118.9 (CH), 119.8 (C), 122.5 (CH), 123.4 (C), 127.2 (CH), 127.8 (CH), 129.2 (CH), 135.9 (C), 134.1 (C), 154.7 (C), 166.5 (C), 170.8 (C), 171.3 (C), 173.7 (C). MS m/z (ESI): 427.2 [$(M - H)^-$], 449.0 [$(M - H)^- + Na$].

3-[[4-[[(2-Carboxyethyl)amino]carbonyl]phenyl]azo]-2-hydroxybenzoic Acid (Balsalazide 3-isomer, 4)

Concentrated hydrochloric acid (10 mL, ~36% w/w) was added to a suspension of *N*-(4-aminobenzoyl) β-alanine (10 g, 0.05 mol) in DM water (145 mL) at 20–25 °C. The obtained clear solution was cooled to 0 to −2 °C, and a solution of sodium nitrite (3.5 g, 0.05 mol) in DM water (20 mL) was added to it. Thus obtained, diazotized solution was added to a solution of salicylic acid (8 g, 0.06 mol) in a water/isopropyl alcohol mixture (1:1, 150 mL) containing sodium carbonate (18 g) at 2 to −2 °C,

maintaining pH $\geq$ 8. After completion of reaction, the reaction mass was acidified, filtered, washed, and dried. Thereafter, the desired impurity balsalazide 3-isomer **4** was purified by flash chromatography (ethyl acetate/methanol as eluant) to get brown solid. (1.2 g, 7% yield). IR (KBr, cm^{-1}): 3426 and 3185 (OH and NH), 1717 and 1711 (C=O), 1631(C=O amide), 1572 and 1545 (C=C aromatic), 1444 and 1399 (aliphatic C-H), 1229 (C-N), 1071 (C-O), 771 and 736 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.52 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 6.61 (dd, J = 7.7 Hz, 1H), 7.57 (dd, J = 8.0 Hz and 1.7 Hz, 1H), 7.86 (d, J = 8.2 Hz, 2H), 7.87 (dd, J = 8.0 Hz and 1.7 Hz, 1H), 8.00 (d, J = 8.5 Hz, 2H), 8.70 (t, J = 5.5 Hz, 1H), 18.43 (brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 35.0 (CH_2), 36.7 (CH_2), 115.1 (CH), 119.3 (CH), 122.8 (CH), 123.5 (C), 129.1 (CH), 135.0 (CH), 136.1 (C), 142.1 (C), 155.0 (C), 165.5 (C), 166.4 (C), 171.4 (C), 174.1 (C). MS m/z (ESI): 356.2 [$(M-H)^-$], 378.0 [$(M-H)^- + Na$].

3-[4-(4-Hydroxy Phenyl Azo)benzoyl Amino]propionic Acid (Decarboxy Balsalazide, 5)

Concentrated hydrochloric acid (20 mL, ~36% w/w) was added to a suspension of *N*-(4-aminobenzoyl)-β-alanine (20 g, 0.1 mol) in DM water (300 mL) at 20–25 °C, and the resulting clear solution was cooled to 2 to −2 °C. To the obtained solution, an aqueous solution of sodium nitrite (6.88 g, 0.1 mole) in DM water (20 mL) was added at 2 to −2 °C. Thus obtained, diazotized solution was added slowly to a precooled solution of 4-hydroxybenzoic acid (13.53 g, 0.1 mole) in DM water (200 mL) containing (8.15 g) of sodium hydroxide and (15.9 g) of sodium carbonate at 2 to −2 °C, maintaining pH $\geq$ 8. After completion of the reaction, the reaction mass was acidified, filtered, washed, and dried to obtain the desired impurity **5** (27.38 g, 91% yield). IR (KBr, cm^{-1}): 3336 (OH and NH), 3048 (Ar-H), 2969 (aliphatic C-H), 1707 and 1606 (C=O), 1589 and 1543 (C=C aromatic), 1464 (aliphatic C-H), 1228 (C-N) 1087 (C-O), 774 and 724 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.54 (m, 2H, CH_2), 3.50 (*m*, 2H, CH_2), 6.97 (d, J = 8.8 Hz, 2H), 7.84 (d, J = 8.8 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 8.04 (d, J = 8.8 Hz, 2H), 8.72 (t, J = 5.5 Hz, 1H), 10.47 (brs, 1H), 12.30 (brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 33.8 (CH_2), 35.7 (CH_2), 116.1 (CH), 121.9 (CH), 125.2 (CH), 128.4 (CH), 135.6 (C), 145.3 (C), 153.6 (C), 161.5 (C), 165.6 (C), 172.9 (C). MS m/z (ESI): 314.0 [$(MH)^+$], 336.0 [$M^+ + Na$].

2,4-bis-[[4-[(2-Carboxyethyl)amino]carbonyl]phenyl]azo Salicylic Acid (bis-Azo Salicylic Acid, 6)

Concentrated hydrochloric acid (20 mL, ~36% w/w) was added to a suspension of *N*-(4-aminobenzoyl)-β-alanine (20 g, 0.1 mol) in DM water (250 mL) at 20–25 °C. The resulting clear solution was cooled to 2 to −2 °C, and sodium nitrite (6.7 g, 0.1 mol) in DM water (30 mL) was added to it. The diazotized solution was added to a precooled solution of salicylic acid (6.6 g, 0.05 mol) in DM water (300 mL) containing sodium hydroxide (7.68 g) and sodium carbonate (15.26 g) at 2 to −2 °C, maintaining pH above 8. After addition, the reaction mass was acidified, filtered, washed with DM water, and dried. Thereafter, the desired impurity was purified by

flash chromatography. IR (KBr, cm^{-1}): 3440 and 3178 (OH and NH), 2968 and 2975 (aliphatic C-H), 1713 and 1631 (C=O), 1548 and 1493 (C=C aromatic), 1451 (aliphatic C-H), 1242 (C-N), 1074 (C-O), 772 and 739 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.47 (m, 4H, 2CH_2), 3.48 (m, 4H, 2CH_2), 7.90 (2d, $J = 8.8$ Hz, 4H), 8.01 (2d, $J = 8.8$ Hz, 4H), 8.15 (d, $J = 2.5$ Hz, 1H), 8.51 (d, $J = 2.5$ Hz, 1H), 8.70 and 8.74 (2 t, $J = 5.2$ Hz, 2H), 18.89 (brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 34.2 (CH_2), 35.9 (CH_2), 113.7 (CH), 121.2 (C), 121.7 (CH), 122.2 (CH), 128.3 (CH), 128.8 (CH), 135.2 (C), 135.6 (C), 139.5 (C), 142.4 (C), 153.7 (C), 154.0 (C), 165.5 (C), 169.5 (C), 171.2 (C), 173.2 (C). MS m/z (ESI): 577.2 [$(MH)^+$], 599.0 [$M^+ + Na$].

5-[2-[4′,5-bis[(2-Carboxyethyl)carbamoyl]biphenyl-2-yl]diazenyl]-2-hydroxybenzoic Acid (Biphenyl-Azo Salicylic Acid, 7)

This related substance was prepared in laboratory as described in previous example. IR (KBr, cm^{-1}): 3446 and 3180 (OH and NH), 2960 and 2971 (aliphatic C-H), 1720 and 1635 (C=O), 1552 and 1490 (C=C aromatic), 1443 (aliphatic C-H), 1248 (C-N), 1076 (C-O), 775 and 731 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.54 (m, 4H, 2CH_2), 3.49 (m, 4H, 2CH_2), 6.70 (d, $J = 8.8$ Hz, 1H), 7.58 (d, $J = 8.5$ Hz, 2H), 7.60 (dd, $J = 8.8$ Hz and 2.5 Hz, 1H), 7.70 (d, $J = 8.8$ Hz, 1H), 7.93 (d, $J = 8.5$ Hz, 2H), 7.97 (dd, $J = 8.8$ Hz and 1.4 Hz, 1H), 8.05 (d, $J = 1.4$ Hz, 1H), 8.17 (d, $J = 2.5$ Hz, 1H), 8.64 (t, $J = 5.2$ Hz, 1H), 8.77 (t, $J = 5.2$ Hz, 1H), 18.34 (brs, 1H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 34.6 (CH_2), 36.5 (CH_2), 116.9 (CH), 119.2 (CH), 119.9 (C), 126.2 (CH), 127.5 (CH), 128.6 (CH), 129.4 (CH), 130.4 (CH), 131.3 (CH), 134.0 (C), 135.6 (C), 138.7 (C), 141.9 (C), 143.5 (C), 151.8 (C), 166.4 (C), 166.8 (C), 171.1 (C), 173.8 (C). MS m/z (ESI): 549.2 [$(MH)^+$], 571.0 [$M^+ + Na$].

3-[4-[5-[4-(2-Carboxy Ethyl Carbamoyl)phenylazo]-2-hydroxy Phenylazo]benzoyl Amino] Propionic Acid (Bis-Azo Diacid, 8)

Concentrated hydrochloric acid (5 mL, ~36% w/w) was added to a suspension of *N*-(4-aminobenzoyl)-β-alanine (5 g, 0.024 mol) in DM water (75 mL) at 20–25 °C, and the resulting solution was cooled to 2 to −2 °C. A solution of sodium nitrite (1.74 g, 0.025 mol) in DM water (10 mL) was added at 2 to −2 °C. The resulting diazotized solution was added to a precooled solution of 4-hydroxybenzoic acid (1.65 g, 0.012 mole) in DM water (100 mL) containing sodium carbonate (9.1 g) at 2 to −2 °C, maintaining pH above 8. After completion of the reaction, the reaction mass was acidified, filtered, washed with DM water, and dried. Thereafter, the obtained product was suspended in aqueous acetic acid (400 mL) and refluxed for 20 min, then cooled to 25–30 °C, filtered, and dried to obtain impurity **8** (4.28 g, 32% yield). IR (KBr, cm^{-1}): 3298 (NH), 3057 (Ar-H), 2921 (aliphatic C-H), 1630 and 1604 (C=O), 1577 and 1538 (C=C aromatic), 1430 (C-H), 1214 (C-N), 1085 (C-O), 799 and 772 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.57 (m, 4H, 2CH_2), 3.52 (m, 4H, 2CH_2), 7.32 (d, $J = 8.8$ Hz, 1H), 7.94 (d, $J = 8.2$ Hz, 1H), 7.95–8.15 (m, 8H), 8.24 (s, 1H), 8.72 and 8.75 (2t, $J = 5.2$ Hz, 2H), 11.54 (brs, 1H), 12.27 (brs, 2H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 33.7 (CH_2),

35.7 (CH_2), 114.1 (CH), 119.3 (CH), 122.2 (CH), 122.8 (CH), 128.0 (CH), 128.4 (CH), 128.5 (CH), 136.1 (C), 136.5 (C), 139.0 (C), 145.1 (C), 153.2 (C), 153.3 (C), 159.0 (C), 165.5 (C), 165.5 (C), 172.9 (C). MS m/z (ESI): 533.3 [$(MH)^+$], 555.1 [$M^+ + Na$].

2,4-bis[[4[[(2-Carboxyethyl)amino]carbonyl]phenyl]azo]-3-[4-[[(2-carboxyethyl)amino]carbonyl]-phenyl]phenol (Bis-Azo Triacid, 9)

N-(4-Aminobenzoyl)-β-alanine (10 g, 0.05 mol) was suspended in DM water (120 mL) at 20–25 °C. Concentrated hydrochloric acid (10 mL, ~36% w/w) was added to the obtained suspension and cooled to 2 to −2 °C. A solution of sodium nitrite (3.5 g, 0.05 mol) in DM water (20 mL) was added at 2 to −2 °C. The resulting diazotized solution was added to a solution of 4-hydroxybenzoic acid (1.65 g, 0.01 mol) in DM water (100 mL) containing sodium carbonate (18 g) at 2 to −2 °C, maintaining pH above 8. After completion of reaction, the temperature of the reaction mass was raised to 35–40 °C and acidified. Thereafter, the precipitated product was filtered, washed, and dried. The obtained product was suspended in ethyl methyl ketone (100 mL) having 10% v/v water and refluxed for 20 min. Thereafter, the suspension was cooled to 20–25 °C, filtered, and dried to obtain product **9** (3.15 g, 36% yield). IR (KBr, cm^{-1}): 3261 (OH and NH), 1714 and 1639 (C=O), 1548 and 1494 (C=C aromatic), 1228 (aliphatic C-N), 1070 (C-O), 789 and 760 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.41 (m, 4H, 2CH_2), 2.47 (m, 2H, CH_2), 3.43 (m, 4H, 2CH_2), 3.49 (m, 2H, CH_2), 6.98 (d, $J = 9.3$ Hz, 1H), 7.43 (d, $J = 8.2$ Hz, 2H), 7.51 (2d, $J = 8.8$ Hz and 2.5 Hz, 4H), 7.87 (d, $J = 8.0$ Hz, 6H), 7.93 (d, $J = 9.3$ Hz, 1H), 8.55, 8.64 and 8.72 (3 t, $J = 5.2$ Hz, 3H). ^{13}C NMR and DEPT (DMSO-d_6, 300 MHz, δ ppm): 37.3 (CH_2), 37.4 (CH_2), 38.4 (CH_2), 121.6 (CH), 123.8 (CH), 124.2 (CH), 124.3 (CH), 127.4 (CH), 130.4 (CH), 130.6 (CH), 134.6 (CH), 135.1 (C), 136.5 (C), 137.7 (C), 138.4 (C), 139.8 (C), 144.3 (C), 146.5 (C), 154.0 (C), 155.7 (C), 158.2 (C), 167.6 (C), 167.8 (C), 168.6 (C), 176.5 (C), 176.8 (C). MS m/z (ESI): 724.1 [$(MH)^+$], 746.2 [$M^+ + Na$].

3-(4-Acetylamino Benzoylamino)-propionic Acid (N-Acetyl-4-ABBA, 10)

N-(4-Aminobenzoyl)-β-alanine (30 g, 0.15 mol) was added to an acetic acid/water mixture (2:1, 90 mL) at 25–30 °C. Acetic anhydride (30 g, 0.29 mol) was added to the obtained suspension, maintaining temperature at 30–45 °C. The reaction mass was stirred at 35–40 °C for 1 h and diluted with acetone (90 mL). The precipitated product was filtered, washed with DM water followed by acetone, and dried to obtain **10** (32.46 g, 90% yield). IR (KBr, cm^{-1}): 3326 and 3196 (OH and NH), 3064 (Ar-H), 2907 (aliphatic C-H), 1706, 1693 and 1626 (C=O), 1590 and 1537 (C=C aromatic), 1429 (aliphatic C-H), 1262 (C-N), 1083 (C-O), 813 and 763 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.06 (s, 3H, CH_3), 2.48 (t, 2H, CH_2), 3.44 (m, 2H, CH_2), 7.62 (d, $J = 8.5$ Hz, 2H), 7.77 (d, $J = 8.5$ Hz, 2H), 8.38 (t, $J = 5.2$ Hz, 1H), 10.13 (s, 1H).

5-Acetamido-2-hydroxybenzoic Acid (5-Acetamido-SA, 11)

5-Aminosalicylic acid (50 g, 0.33 mol) was added to an acetic acid/acetic anhydride mixture (1:1, 100 mL), stirred at reflux for 30 min, and cooled to 20–25 °C. The

precipitated product was filtered, washed with DM water, suspended in 10% w/w aqueous sodium hydroxide solution, and stirred for 1 h at 25–30 °C. The pH was adjusted to 2 to precipitate the product. The product **11** was filtered, washed with DM water, and dried (59.7 g, 94% yield). IR (KBr, cm^{-1}): 3357 and 3106 (OH and NH), 2874 (aliphatic C-H), 1685 and 1603 (C=O), 1539 and 1516 (C=C aromatic), 1420 (aliphatic C-H), 1238 (C-N), 807 and 792 (Ar-H out of plane bend). ^{1}H NMR (DMSO-d_6, 300 MHz, δ ppm): 2.14 (s, 3H, CH_3), 7.07 (dd, $J = 8.78$ Hz and 1.92 Hz, 1H), 7.32 (d, $J = 1.92$ Hz, 1H), 7.74 (d, $J = 8.78$ Hz, 1H), 9.60 (s, 1H), 11.32 (brs, 1H).

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