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RESEARCH ARTICLE

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Synthesis of stable-isotope-labeled *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and *N*-(3-dimethylaminopropyl)-*N*′-ethylurea

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Summary

N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) is a carbodiimide coupling reagent commonly used for the preparation of amides from carboxylic acids and amines. Because of initial concerns regarding the genotoxicity of EDC and its use in GMP syntheses at Bristol Myers Squibb, the quantitation of residual EDC and its by-product *N*-(3-dimethylaminopropyl)-*N*′-ethylurea (EDU) by liquid chromatography–mass spectrometry (LCMS) impurity analysis was required. These analyses required the use of stable-isotope-labeled EDC and EDU to serve as internal standards. To meet this need, stable-isotope-labeled EDC **9** and EDU **10** were prepared from [1,2-$^{13}C_2$] ethylene glycol and [^{13}C,^{15}N] potassium cyanide in overall yields of 6% and 8%, respectively.

KEYWORDS

EDC, EDU, labeling, stable isotope

1 | INTRODUCTION

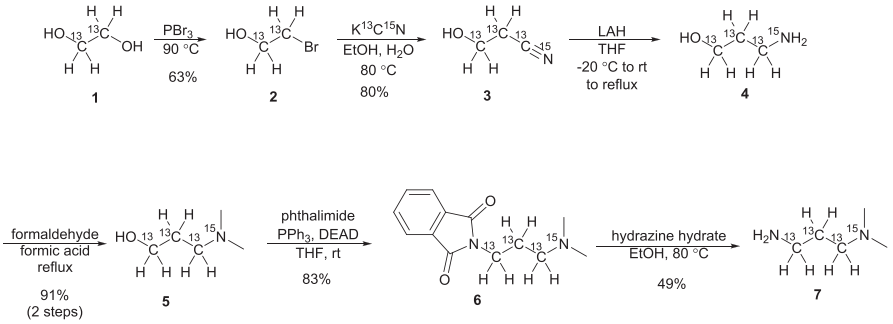
Amide bond formation reactions are one of the most common reaction types in pharmaceutical chemistry. A 2011 analysis of publications from the medicinal chemistry departments of AstraZeneca, GlaxoSmithKline, and Pfizer revealed that amide bond formation reactions accounted for 16% of the reactions in the dataset.[1] The classic method for amide bond formation involves the condensation of carboxylic acids and amines mediated by a stoichiometric coupling reagent. These coupling agents were the subject of an extensive review.[2]

The role of the coupling agent is to convert the carboxylic acid into a more reactive ester intermediate. The reactive ester is then attacked by the amine to generate the amide or an additive such as 1-hydroxybenzotriazole (HOBt) that improves the reaction efficiency. Carbodiimides are often selected to serve as the coupling reagent for amide bond formation. The carboxylic acid is activated for substitution when the oxygen of the acid attacks the central carbon of the carbodiimide. In this process, the hydroxyl group is essentially converted to a stronger leaving group that is ultimately expelled by a nucleophile to generate the amide and a urea byproduct.

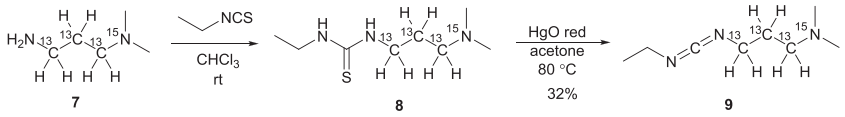
Among the carbodiimides available, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) is commonly used because it is available as an HCl salt, is easy to handle, and the urea by-product, *N*-(3-dimethylaminopropyl)-*N*′-ethylurea (EDU), is soluble in water and can easily be removed.[3] At the time of this work, EDC was considered to be potentially genotoxic; however, later studies determined that EDC is not genotoxic.[4] in vitro Ames bacterial mutagenicity tests indicated EDC is genotoxic, but the reagent is not genotoxic in vivo when administered to rats orally. When administered orally, EDC is hydrolyzed to EDU, which is not genotoxic.

EDC has been used in the synthesis of several compounds under clinical development at Bristol Myers Squibb. Because of prior concerns about potential genotoxicity, quantitation of residual EDC and EDU in

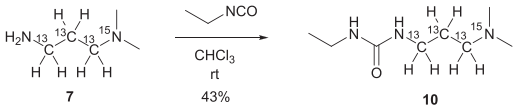
SCHEME 1 Synthesis of [1,2,3-$^{13}C_3$] 3-dimethyl [^{15}N]aminopropylamine (**7**)



SCHEME 2 Synthesis of *N*-([1,2,3-$^{13}C_3$] 3-dimethyl[^{15}N]aminopropyl)-*N*′-ethylcarbodiimide (**9**, EDC)



active pharmaceutical ingredients (APIs) using liquid chromatography–mass spectrometry (LCMS) impurity analysis was needed. The analyses utilized stable-isotope-labeled internal standards of both EDC and EDU. The syntheses of these labeled compounds are described below.



SCHEME 3 Synthesis of 1-ethyl-3([1,2 3-$^{13}C_3$]3-dimethyl[^{15}N] aminopropyl)urea (**10**, EDU)

2 | RESULTS AND DISCUSSION

The synthetic strategy involved preparing compound **7**, a labeled diamine intermediate that can be elaborated to either EDC or EDU, from commercially available [1,2-$^{13}C_2$] ethylene glycol **1** (Scheme 1). The first steps of the synthesis were based on the known synthesis of [D_6] 3-dimethylaminopropyl alcohol.[5] Compound **1** was heated with phosphorous tribromide to give bromoethanol **2** in 63% yield. Compound **2** was reacted with [^{13}C^{15}N] potassium cyanide to yield [$^{13}C_3$^{15}N] ethylene cyanohydrin (**3**). The nitrile of compound **3** was reduced to the amine using lithium aluminum hydride to yield hydroxylamine **4**. An initial attempt to purify compound **4** via vacuum distillation was not successful mainly because of material loss from vacuum distillation on a small scale. To limit material loss, compound **4** was carried forward to the next step as a crude product. Methylation of compound **4** with formaldehyde and formic acid followed by Vigreux distillation yielded compound **5** containing residual diethyl ether. Mitsunobu reaction with phthalimide was followed by removal of the phthaloyl group with hydrazine to yield compound **7**. Compound **7** was isolated via Vigreux distillation and contained residual diethyl ether.

Following the work of Pouyani et al.,[6] the reaction of [1,2,3-$^{13}C_3$] 3-dimethyl [^{15}N]aminopropylamine (**7**) with ethyl isothiocyanate in chloroform provided thiourea **8** in quantitative yield (Scheme 2). Thiourea **8** was subjected to dehydrosulfurization using mercury oxide red in refluxing acetone, followed by microdistillation to give the desired *N*-([1,2,3-$^{13}C_3$] 3-dimethyl[^{15}N]aminopropyl)-*N*′-ethylcarbodiimide (**9**, EDC) in 32% yield, which was used in a free base form as an internal standard to quantify residual EDC in APIs.

The synthesis of 1-ethyl-3([1,2 3-$^{13}C_3$] 3-dimethyl[^{15}N] aminopropyl)urea (**10**, EDU) was accomplished by reacting [1,2,3-$^{13}C_3$] 3-dimethyl [^{15}N]aminopropylamine (**7**) with ethyl isocyanate in chloroform followed by reverse-phase preparative high-performance liquid chromatography (HPLC) (Scheme 3).

3 | CONCLUSION

In order to quantify the amount of residual EDC and EDU in clinical APIs, stable-isotope-labeled EDC (**9**) and EDU (**10**) were synthesized. The labeled starting materials were [$^{13}C_2$] ethylene glycol and [^{13}C,^{15}N] potassium cyanide. The labeled compounds were prepared in 6% overall yield for [$^{13}C_3$,^{15}N] EDC (**9**) and 8% yield for [$^{13}C_3$,^{15}N] EDU (**10**). One of the primary challenges of these syntheses was the volatile nature of the synthetic intermediates. This

challenge was overcome by a combination of telescoping one intermediate, using Vigreux distillation, and handling the compounds as concentrated solutions in diethyl ether where needed. Ultimately, the synthesis successfully delivered stable labeled standards of EDC and EDU that were critical for the quantitation of these impurities in clinical samples.

4 | EXPERIMENTAL

4.1 | General

All reagents and solvents used were of ACS grade or higher. The reactions were conducted under an atmosphere of nitrogen unless specified differently. Ethylene-$^{13}C_2$ glycol, 99 atom% ^{13}C, and potassium cyanide-^{13}C-^{15}N, 99 atom% ^{13}C & ^{15}N were purchased from Isotec, Inc., Miamisburg, OH. LCMS data were obtained on a Thermo XLQ 2.0 Mass Spectrometer System with electrospray ionization. ^1H NMR spectra were recorded on a Bruker 400 MHz Avance II NMR spectrometer and ^{13}C NMR on a Bruker Avance II 300 MHz spectrometer with Ultrashield™ magnet. Preparative HPLC was performed on a Varian HPLC system with two PrepStar 218 pumps and ProStar 320 variable UV detector. Preparative HPLC method: Phenomenex LUNA C18, 5 μm, 21.2 × 250 mm, UV detection at 220 nm, flow rate 20 ml/min. Mobile phase A: 10 mM NH_4HCO_3, mobile phase B: acetonitrile. Gradient: 15%–50% B over 10 min.

4.1.1 | [1,2-$^{13}C_2$]2-Bromoethanol (2)

To neat [1, 2-$^{13}C_2$]ethylene glycol **1** (5 g, 78 mmol) was added phosphorous tribromide (2.43 ml, 25.8 mmol). The mixture was then heated to 90°C for 6 h. Vacuum distillation through a 2″ Vigreux column at 65°C–70°C/10 mmHg yielded [1,2-$^{13}C_2$]2-bromoethanol (**2**) (6.2 g, 63% yield). ^1H NMR (400 MHz, CD_3CN) δ 3.95 (m, 1H), 3.65 (m, 1H), 3.59 (m, 1H), 3.28 (m, 1H). The ^1H NMR data are consistent with those of the authentic 2-bromoethanol: δ 3.77 (t, J = 5.9 Hz, 2H), 3.47 (t, J = 5.8 Hz, 2H). ^{13}C NMR (100 MHz, CD_3CN) δ 63.72, 63.42, 36.65, 36.34.

4.1.2 | [$^{13}C_3$,^{15}N]Ethylene cyanohydrin (3)

To a solution of [1,2-$^{13}C_2$]2-bromoethanol (**2**) (6.2 g, 49 mmol) in ethanol (16 ml) was added potassium cyanide-^{13}C-^{15}N (3.29 g, 49 mmol) in water (4.8 ml). Upon addition, the solution became turbid. The mixture was heated at 80°C for 6 h then cooled in an ice bath and filtered. The filtrate was concentrated under reduced pressure at 40°C. The resulting solution was neutralized with 1 N HCl and diluted with acetone. The resulting precipitate was removed by filtration and washed with acetone. The filtrate and acetone wash were pooled and concentrated under reduced pressure at 40°C. The residue was then vacuum distilled at 83°C–85°C/1.5 mmHg to yield [1,2-$^{13}C_2$] ethylene cyanohydrin (**3**) (2.95 g, 80% yield). ^1H NMR (400 MHz, CD_3CN) δ 3.69 (dddd, J = 146.1 Hz, 12.6 Hz, 6.3 Hz, 1.7 Hz, 2H), 2.70 (m, 1H), 2.37 (m, 1H). The ^1H NMR data are consistent with those of the unlabelled ethylene cyanohydrin: δ 3.69 (t, J = 6.1 Hz, 2H), 2.53 (t, J = 6.2 Hz, 2H). ^{13}C NMR(100 MHz, CD_3CN) δ 120.03 (ddd, J = 3.8 Hz, 17.2 Hz, 56.3 Hz), 58.22 (dd, 3.8 Hz, 36.3 Hz), 22.01 (ddd, J = 2.8 Hz, 36.3 Hz, 56.3 Hz).

4.1.3 | [1,2,3-$^{13}C_3$] 3-[^{15}N] Aminopropanol (4)

[1,2-$^{13}C_2$]Ethylene cyanohydrin (**3**) (2.95 g, 39.3 mmol) in THF (60 ml) was cooled to −20°C. Lithium aluminum hydride (1 M in THF) was added dropwise to the mixture. Following lithium aluminum hydride addition, the reaction mixture was warmed to room temperature and then heated at reflux for 8 h. The mixture was cooled to room temperature and then quenched by the sequential addition of water (3 ml), 15% NaOH (3 ml), and water (9 ml). During the reaction quench, a solid formed. The solid was removed by filtration and washed with THF (3 × 10 ml). The filtrate and THF wash were combined and concentrated under reduced pressure in a rotary evaporator (stop with about 10 ml left). The crude product was used without further purification in the subsequent reaction. ^1H NMR (400 MHz, CD_3CN) δ 3.61 (dddd, J = 46.2 Hz, 2.4 Hz, 5.8 Hz, 11.4 Hz, 1H), 2.89 (m, 1H), 2.62 (m, 1H), 1.68 (m, 1H), 1.43 (m, 1H). ^1H NMR data are consistent with unlabelled 3-aminopropanol: δ 3.61 (t, J = 5.9 Hz, 2H), 2.76 (t, J = 6.4 Hz, 2H), 1.56 (tt, J = 6.4, 5.9 Hz, 2H). ^{13}C NMR (100 MHz, CD_3CN) δ 62.47 (d, J = 36.3 Hz), 41.28 (dd, J = 36.2 Hz, 3.8 Hz), 35.95 (t, J = 36.7 Hz). MS (ES^+) m/z: 79.92 $[M + H]^+$.

4.1.4 | [1,2,3-$^{13}C_3$]3-Dimethyl[^{15}N] aminopropanol (5)

[1,2,3-$^{13}C_3$]3-[^{15}N]Aminopropanol (**4**) from the previous experiment was dissolved in formic acid (11.2 ml), cooled in ice-water. Formaldehyde solution (37%, 11.2 ml) was added, and the reaction mixture was heated under reflux for 12 h. Thirty-seven percent aqueous hydrochloric acid

(10 ml) was added to the reaction mixture, which was then evaporated to dryness under reduced pressure. Excess 50% aq. NaOH solution was added to the residue, and the product was extracted with diethyl ether (4 × 20 ml). The ether solution was dried over Na_2SO_4, filtered, and distilled with a 2″ Vigreux column at atmospheric pressure at 58°C. The distillation was stopped with about 8 ml collected in the flask. The distilled product (5.2 g) was used without further purification in the subsequent reaction. ^{1}H NMR showed a mixture of the desired product and diethyl ether with a molar ratio of 1.98:1 (calculated 74% purity, 91% yield from compound **3**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (dddd, J = 41.5 Hz,10.9 Hz, 6.0 Hz, 2.1 Hz, 2H), 2.69 (m, 1H), 2.42 (m, 1H), 2.28 (d, J = 5.3 Hz, 6H),1.83 (m, 1H), 1.58 (m, 1H). ^{1}H NMR data are consistent with unlabelled 3-dimethylaminopropanol: δ 3.81 (t, 5.3 Hz, 2H), 2.54 (t, J = 5.9 Hz, 2H), 2.27 (s, 6H), 1.70 (tt, J = 6.0 Hz, 5.5 Hz, 2H). ^{13}C NMR(400 MHz, $CDCl_3$) δ 64.71 (dd, J = 35.3 Hz, 1.9 Hz), 60.17 (ddd, J = 38.2 Hz, 2.8 Hz, 2.9 Hz), 27.89 (t, J = 37.2 Hz). MS (ES^+) m/z: 108.00 $[M + H]^+$.

4.1.5 | 2-([1,2,3-$^{13}C_3$]3-Dimethyl[^{15}N] aminopropyl)isoindoline-1,3-dione (6)

To an ice-cold mixture of [1,2,3-$^{13}C_3$]3-dimethyl[^{15}N] aminopropanol (**5**) (1.630 g, 11.3 mmol, 74%), phthalimide (2.69 g, 18.30 mmol), and triphenylphosphine (4.80 g, 18.30 mmol) in tetrahydrofuran (47.2 ml) was added diethyl azodicarboxylate (8.33 ml, 18.30 mmol) (40% in toluene) over 10 min. The reaction was slowly brought to room temperature and stirred overnight. The solvent was evaporated under reduced pressure, and the crude product was purified by flash chromatography with ethyl acetate and hexanes to yield the desired product **6** (2.2 g, 83% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, J = 3.0 Hz, 5.5 Hz, 2H), 7.71 (dd, J = 3.0 Hz, 5.6 Hz, 2H), 3.74 (dddd, J = 40.2 Hz, 3.6 Hz, 7.0 Hz, 10.6 Hz, 2H), 2.35 (dddd, J = 31.7 Hz, 4.2 Hz, 7.5 Hz, 11.1 Hz, 2H), 2.21 (d, J = 5.2 Hz, 6H), 1.97 (m, 1H), 1.72 (m, 1H). ^{1}H NMR data are consistent with unlabelled 2-(3-dimethyl aminopropyl)isoindoline-1,3-dione: δ 7.85 (dd, J = 3.0 Hz, 5.5 Hz, 2H), 7.71 (dd, J = 3.1 Hz, 5.3 Hz, 2H), 3.75 (t, J = 7.3 Hz, 2H), 2.35 (t, J = 7.3 Hz, 2H), 2.22 (s, 6H), 1.85 (tt, J = 7.5 Hz, 7.5 Hz, 2H). ^{13}C NMR(400 MHz, $CDCl_3$) δ 57.02 (dd, J = 3.8 Hz, 38.1 Hz), 36.33 (dd, J = 36.3 Hz, 1.9 Hz), 26.64 (ddd, J = 36.9 Hz, 39.3 Hz, 2.4 Hz).

4.1.6 | [1,2,3-$^{13}C_3$]3-Dimethyl[^{15}N] aminopropylamine (7)

To 2-([1,2,3-$^{13}C_3$]3-dimethyl [^{15}N]aminopropyl)-isoindoline-1,3-dione (**6**) (2.2 g, 7.45 mmol) in ethanol (143 ml) was added hydrazine monohydrate (3.61 ml, 74.5 mmol). The mixture was stirred at 80°C for 12 h. The phthalimide cleavage by-product precipitated from solution during the reaction and was filtered. After drying, the isolated solid weighed 0.91 g, indicating that approximately 0.6 g of the desired amine was formed. To the ethanol filtrate was added 1 N aq. hydrochloric acid and stirred for 5 min. The solvent was then evaporated under reduced pressure to dryness. Excess 50% aqueous NaOH (10 ml) was added with cooling, and the mixture was extracted with diethyl ether (4 × 20 ml). The ether extracts were dried over anhydrous Na_2SO_4 and filtered. The ether solution was distilled with a 2″ Vigreux column at atmospheric pressure and heating at 58°C to a volume of 2–3 ml to yield compound **7** as a solution in diethyl ether. The mass of the product solution was 1.51 g. Based on ^{1}H NMR integration, the molar ratio of product amine versus ether is 1: 4.1, 25.8% amine by weight. The yield of compound **7** was therefore calculated to be 0.39 g (49% yield). The product solution was used without further purification. ^{1}H NMR (400 MHz, CD_3OD) δ 2.84 (m, 1H), 2.51–2.59 (m, 2H), 2.30 (dd, J = 5.0 Hz, 6H), 2.27 (m, 1H), 1.83 (m, 1H), 1.57 (m, 1H). The ^{1}H NMR data are consistent with those of the unlabelled 3-dimethyl aminopropylamine: δ 2.65 (t, J = 7.2 Hz, 2H), 2.36 (t, J = 7.7 Hz, 2H), 2.24 (s, 6H), 1.65 (m, 2H). ^{13}C NMR (400 MHz, CD_3OD) δ 58.45 (dd, J = 3.8 Hz, 38.2 Hz), 40.97 (dd, J = 1.9 Hz, 36.2 Hz), 31.44 (ddd, J = 1.9 Hz, 36.3 Hz, 37.3 Hz).

4.1.7 | *N*-([1,2,3-$^{13}C_3$]3-Dimethyl[^{15}N] aminopropyl)-*N*′-ethylcarbodiimide (9)

An ether solution of [1,2,3-$^{13}C_3$]3-dimethyl[^{15}N] aminopropylamine (**7**) (4.3 g, 21.4% amine by mass, 0.91 g amine, 8.57 mmol) and ethyl isothiocyanate in chloroform (5 ml) were stirred at room temperature overnight under Ar. The solvent was evaporated under a gentle stream of nitrogen to yield compound **8**, which was not purified. The crude material was dissolved in acetone (11.4 ml), mercury (II) oxide red (2.04 g, 9.43 mmol) was added to the solution, and the mixture was heated at 80°C for 3 h. The bright orange suspension turned black during the course of the reaction, indicating the formation of HgS. The reaction was filtered through Celite, concentrated, and dissolved in acetone. The acetone solution was dried with anhydrous Na_2SO_4, filtered, and transferred to a microdistillation apparatus. Vacuum distillation at 0.6 mmHg/55°C afforded compound **9** (0.44 g, 32% yield) as a colorless liquid. ^{1}H NMR (400 MHz, CD_3OD) δ 3.40 (m, 1H), 3.23 (dq, J = 1.4 Hz, 7.3 Hz, 2H), 3.12 (m, 1H), 2.51 (m, 1H), 2.28 (m, 1H), 2.25 (d, J = 4.7 Hz, 6H), 1.15 (m, 1H), 1.61 (m, 1H), 1.22 (t, 7.2 Hz). ^{1}H NMR data are consistent with unlabeled

N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide: δ 3.24-3.31 (m, 4H), 2.43 (t, J = 7.7 Hz, 2H), 2.28 (s, 6H), 1.77 (m, 2H), 1.25 (t, J = 7.3 Hz, 3H). ^{13}C NMR (400 MHz, CD_3OD) δ 58.06 (dd, J = 38. 1 Hz, 3.8 Hz), 45.86 (dd, J = 37.2 Hz, 2.9 Hz), 30.11 (t, J = 37.2 Hz). MS (ES^+) *m/z*: 160 $[M + H]^+$, 178 $[M + NH_4]^+$. Isotopic distribution, M + 0(156): 0.02%, M + 1(157): 0.02%, M + 2(158): 0.12%, M + 3(159): 2.0%, M + 4(160): 98.0%.

4.1.8 | 1-Ethyl-3([1,2,3-$^{13}C_3$]3-dimethyl[^{15}N]aminopropyl)urea (10)

To a solution of [1,2,3-$^{13}C_3$]3-dimethyl-[^{15}N]aminopropylamine (**7**) (0.39 g, 25.8%, 0.94 mmol) in chloroform (1 ml) was added ethyl isocyanate (67 mg, 0.94 mmol). The mixture was stirred overnight, and the solvent was evaporated under a steady stream of nitrogen. The crude material was purified by preparative HPLC. Column: Phenomenex LUNA C18 5 µm, 21.2 mm × 250 mm, 220 nm, 20 ml/min, solvent A: 10mM NH_4HCO_3, solvent B: acetonitrile, 15%–50% B over 10 min. The collected fractions were checked by LCMS to identify the fractions containing the desired product. The desired product could not be readily detected by UV on the HPLC chromatogram. The product was found in fractions collected between 2 and 7 min. The pooled fractions were evaporated under reduced pressure and then dried under high vacuum to yield compound **10** as a colorless oil (75 mg, 43% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 3.27 (m, 1H), 3.13 (q, J = 7.2 Hz, 2H), 2.98 (m, 1H), 2.51 (m, 1H), 2.27 (d, J = 4.7 Hz, 6H), 2.24 (m, 1H), 1.79 (m, 1H), 1.53 (m, 1H), 1.09 (t, J = 7.2 Hz). ^{13}C NMR (400 MHz, CD_3OD) δ 58.19 (dd, J = 4.3 Hz, 37.7 Hz), 39.29 (dd, J = 1.9 Hz, 37.2 Hz), 29.12 (t, J = 37.2 Hz). MS (ES^+) *m/z*: 178 $[M + H]^+$. Isotopic distribution, M + 0(174): <0.01%, M + 1(175): 0.01%, M + 2(176): 0.15%, M + 3(177): 1.6%, M + 4(178): 98.3%.

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