

Determination of the Source of the *N*-Methyl Impurity in the Synthesis of Pemetrexed Disodium Heptahydrate

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Abstract:

The synthesis of Pemetrexed Disodium Heptahydrate has consistently resulted in a very low level (ca. 0.02%) unknown impurity. To ensure long-term control, the identity and source of the impurity were desired. Isolation and characterization identified the impurity as the *N*-methyl derivative. The source was identified as the methyl groups on the peptide coupling agent, 2,6-Dimethoxy-1,3,5-triazine (CDMT). Further work assured the current conditions provide adequate control.

Introduction:

Pemetrexed Disodium Heptahydrate (LY231514*2Na*7-H₂O, active ingredient in Alimta) (**1**, Figure 1) is a multi-targeted antifolate approved for treatment of mesothelioma and for second-line treatment of nonsmall cell lung cancer (NSCL). Alimta is also under investigation for multiple other cancers. The current amount of Alimta administered to an average size individual (1.8 m²) at the recommended dose (500 mg/m²) is 0.9 g/day. Of particular concern to this work is the possibility of dose escalation during the treatment of future cancers. The International Conference on Harmonization (ICH)¹ sets a high standard for the purity of drug substances. If the dose is less than 2 g/day, impurities over 0.10% are expected to be identified, justified by toxicology data, and controlled to a specification. If the dose exceeds 2 g/day, the qualification threshold is lowered to 0.05%. The synthesis of Pemetrexed Disodium Heptahydrate is very capable of meeting the 0.10% threshold.

All impurities that could exceed that 0.10% threshold have been identified, have been qualified, and are controlled. These impurities are all the result of trace degradation of Pemetrexed Disodium Heptahydrate in the final isolation by oxidative pathways. If Alimta, in the future, were to be investigated at doses requiring the 0.05% threshold, an impurity, which had not been identified at the outset of this work, would require reinvestigation. Levels of this impurity have consistently been ca. 0.02% in both plant and laboratory samples. It was of interest to identify this impurity, determine the source, and ensure that the current control strategy is adequate or to modify the strategy if needed. This report demonstrates the level of scientific understanding needed to ensure the quality of a drug substance.

The commercial synthesis is shown in Scheme 1. The preparation of bisulfite adduct **2** and the conditions under

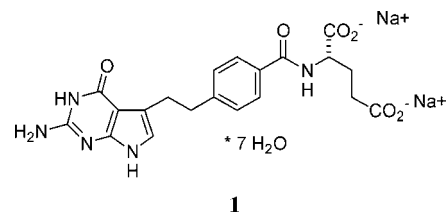


Figure 1. Structure of Pemetrexed Disodium Heptahydrate.

which it is cleaved to the aldehyde have been disclosed previously.² The route from the α -bromide **3** onward reflects further process development on a previously disclosed route.³ The cyclocondensation of **3** with 2,4-diamino-6-hydroxypyrimidine **4** is a very efficient method to construct the pyrrolo-[2,3-*d*] heterocycle. However the product **5** of this step is relatively impure for a pharmaceutical intermediate.

The specifications for **5** allow up to 10 area % impurities by HPLC, with typical levels approaching that limit. Considerable effort failed to discover an effective recrystallization of **5**. The saponification to **6** only provides slight purification, typically less than 1% reduction in total impurities. It is unusual for the specification of isolated intermediates within the pharmaceutical industry to allow these amounts of impurities.⁴ However the specifications are justified, as the remaining steps provide a highly effective purification strategy that includes four isolations under disparate conditions. The acid is activated for coupling by reaction with 2-chloro-4,6-dimethoxytriazine (CDMT)⁵ to form **7** and then reacted with L-glutamic acid diethyl ester (LGADE) **8**. The product of the peptide coupling **9** is isolated as a *p*-toluenesulfonic acid salt **10** from ethanol and then recrystallized from DMSO/EtOH. Compound **10** is then saponified to produce the free acid form of the drug substance (**11**), which is isolated. Finally, the pH is adjusted, and the crystalline disodium salt is isolated as the heptahydrate **1**. The purification strategy has proven very effective; no identified impurity expected in the drug substance (at the outset of this work) was related to the method of synthesis. All impurities expected were the result of trace degradation of **1**.

(2) Kjell, D. P.; Slattery, B. J.; Semo, M. J. *J. Org. Chem.* **1999**, *64*, 5722–5724.

(3) Barnett, C. J.; Wilson, T. M.; Kobierski, M. E. *Org. Proc. Res. Dev.* **1999**, *3*, 184–188.

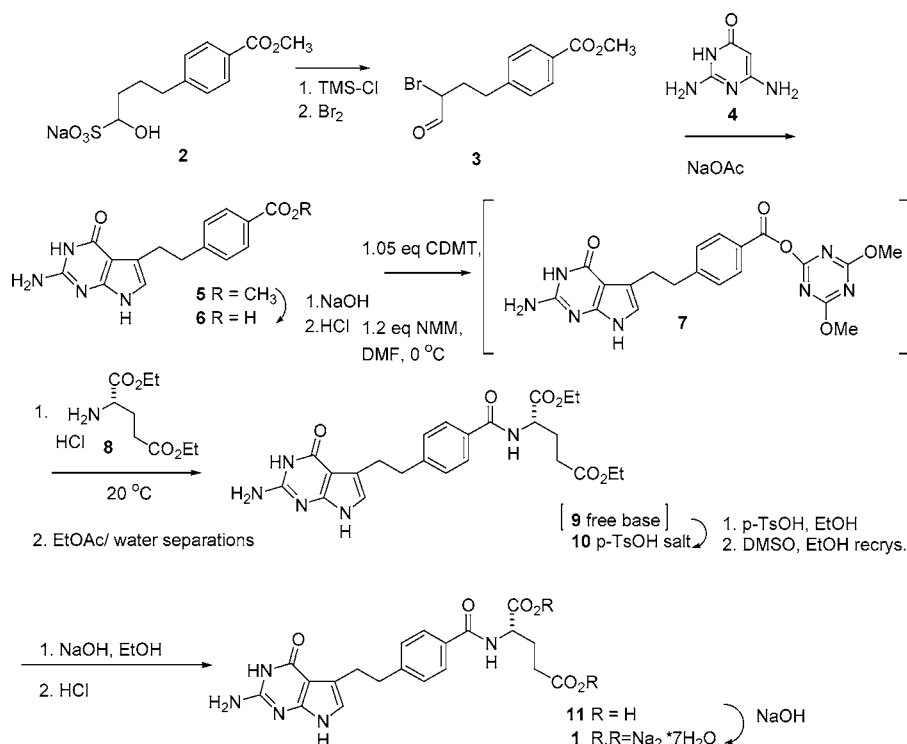
(4) Based on an informal survey, and personal experience, <2–3% total related substances would be a more typical specification for an early intermediate.

(5) Kaminski, Z. J. *Tetrahedron Lett.* **1985**, *26*, 2901–2904.

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(1) International Conference on Harmonisation (ICH) Guidelines, Q3A(R), Impurities in New Drug Substances (Revised Guideline), 2002.

Scheme 1. Commercial synthesis of Pemetrexed Disodium Heptahydrate



Results and Discussion

A key portion of the development of a commercial synthesis is the identification of all impurities in the drug substance and later intermediates. From the outset, identification of the unknown impurity had proven problematic. No samples of Pemetrexed with >0.05% of the impurity were available. Therefore, only a weak, noisy spectrum could be obtained by HPLC/MS. Based on this spectrum a molecular weight of 504 was proposed, but we were unable to draw a structure to fit this mass. Without a structure it is impossible to determine the source of an impurity in a complex synthetic route; the mass expected for earlier steps cannot be predicted. Given the possibility of a higher dose requiring the lower qualification threshold, identification was again attempted. Improved isolation equipment and NMR techniques resulted in the successful identification of the impurity.

Critical to the success of this identification attempt was a change in strategy. Rather than attempt to identify the very low level impurity in situ, the impurity was isolated by preparative chromatography. This allowed an exact mass to be determined, as well as 2D-NMR to be gathered. The initial mass proposed was proven incorrect, and a correct exact mass for the $M + 1$ of 442.1704 was determined. The structure was assigned as the *N*-methyl derivative **12** (Figure 2) and confirmed by independent synthesis. The mass, ^1H , and ^{13}C NMR clearly indicate an *N*-methyl substitution. The HMBC spectrum shows the long range ^1H to ^{13}C correlations which support structure **12** over the other possible *N*-methyl derivatives. Treatment of the drug substance **1** with an excess of methyl iodide, saponification and preparative chromatography prepared **12** in a 65% yield. The spectra of synthetic and isolated **12** matched.

The identification of the *N*-methyl derivative **12** was critical to identification of the source. The first method

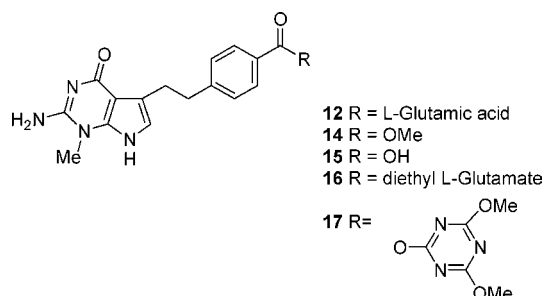


Figure 2. Possible *N*-methyl derivatives.

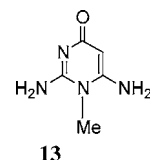


Figure 3.

attempted was to examine all isolated process intermediates for an “ $M + 14$ ” impurity. The results of this examination were ambiguous at best. It was impossible to definitively discern at what point the *N*-methylation occurred.

The second approach pursued was to start at the earliest possible source. Perhaps the *N*-methyl arrives as an impurity in the starting material, 2,4-diamino-6-hydroxypyrimidine **4**. An authentic sample of the *N*-methyl derivative **13** (Figure 3) was prepared⁶ and found to be completely unreactive with the α -bromoaldehyde **3** under the standard conditions and under all alternatives attempted. The limited solubility of **13** contributes to the lack of reactivity. Therefore, the presence of **13** as an impurity in **4**, while not rigorously disproven, could not be causal for *N*-methylation.

(6) Munesada, K.; Suga, T. *J. Org. Chem.* **1987**, 52, 5655–5662.

Table 1. HPLC data for area % *N*-methyl **16** to main peak in standard reaction (**6** to **9**)

conditions	16 (%) at 1 h	prolonged (%)
1.35 equiv of CDMT	0.02	0.09 @ 20h
1.35 equiv of CDMT, DBU	detected ^a	
2.70 equiv of CDMT	0.02	0.3 @ 5 days 0.6 @ 12 days
1.1 equiv of CDMT	0.01	0.01 @ 20 h
2.70 equiv of Aldrich CDMT	0.02	0.18 @ 20 h

^a Yield of **9** poor, complicated mixture produced.

Next we introduced the methyl at the first possible intermediate. Treatment of the cyclocondensation product **5** with methyl iodide again produced the *N*-methyl derivative **14** (Figure 2). An authentic sample allowed us to check production samples of **5** for the impurity by HPLC/MS retention time and mass match. This unambiguously established that **14** was not present in **5** produced in the plant.

A sample of **5** with 5 mass % of the *N*-methyl **14** was then prepared and carried forward through the synthesis in the laboratory. Isolated intermediates (**6**, **10**, **11**, **1**) were examined for impurities with a mass and retention time match. The first match was observed in the p-TsOH salt **10**. The reason for the ambiguity of the first attempt to locate the source (by HPLC/MS without authentic samples) became clear. The amount of the *N*-methyl impurity is very small and was poorly resolved by the original HPLC/MS method from a somewhat larger impurity.

Attention therefore focused on the chemistry converting the acid **6** into the peptide-coupled product **10**. Production samples were used to further narrow the source. The first step within this chemistry is reaction of a slight excess of CDMT with the acid **6** catalyzed by *N*-methylmorpholine (NMM) in DMF at 0 °C. This active ester intermediate **7** is unstable, so it could not be sampled for this purpose. L-Glutamic acid HCl (LGADE) **8** is then added, and the mixture is warmed to 20–25 °C, stirred for 1 h to form **9**, and then sampled. Other sampling points chosen were as follows: the organic phases during the EtOAc/water partitions used to attenuate the DMF levels to allow crystallization, the wet cake of **10** prior to recrystallization, the DMSO solution of **10** as it is dissolved for the recrystallization, and the isolated intermediate itself. The *N*-methyl **16** was detected in all samples, indicating that *N*-methylation occurs during the reaction rather than during isolation, dissolution into DMSO, or recrystallization.

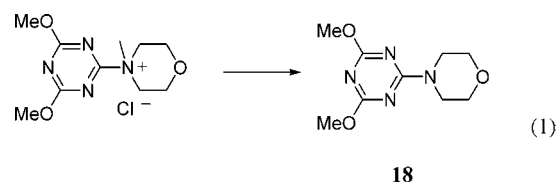
It was then of interest to determine if the *N*-methylation occurs during the activation with CDMT at 0 °C, or during the coupling with LGADE **8**. Laboratory reactions were run using typical and *N*-methyl spiked **6** and then analyzed immediately. The *N*-methyl active ester **17** was detected in the spiked sample but not in the reaction using typical **6**.

Three potential sources of the methyl appear in the peptide chemistry step. *N*-methylmorpholine (NMM), CDMT, and DMF all possess methyl groups. To determine which of these methyls was the source, HPLC of laboratory reaction mixture samples was used. The mixtures are heterogeneous, which makes consistent sampling difficult. To correct for this, the

results were determined as area % *N*-methyl **16** relative to the desired product. Each sample was injected once at a high concentration to get the area of **16** and then diluted 1/100 to determine the area of **9** without saturating the detector. The results of the experiments using this methodology are reported in Table 1. To ensure worst case results, the excess of CDMT was increased from the typical 5% to 35%.

Control reactions under these conditions showed 0.02% **16** if the mixture was sampled after 1 h. Slightly more *N*-methyl was observed on extremely prolonged stirring (0.09% after 20 h).

It is known that the complex of CDMT and NMM decomposes to lose the methyl group originally attached to the NMM, although a rate of decomposition is not reported in DMF (eq 1).⁷



Therefore, the first possibility explored was delivery of the methyl from NMM to form **16**. Optimally, labeled NMM would have been used. As this was not readily available an alternative test was devised; NMM was removed from the system, and DBU was used as the base.⁸ The yield of **9** with this base was very poor, so quantitation as described above is not appropriate, but it was clear that some amount of *N*-methyl **16** was formed. To avoid the problem of poor yield with DBU, a different test was devised. Isolated **10** was treated with CDMT and >2 equiv of base under relatively extreme conditions (100 °C). These results are reported in Table 2. The control reaction with NMM produced 0.6%

Table 2. Treatment of Isolated **10** with 1.35 equiv of CDMT and base at 100 °C

conditions	16 (%) at 1 h	prolonged (%)
CDMT, NMM	0.6	1.4 @ 20 h 1.8 @ 2 days 2.3 @ 5 days
CDMT, DBU	1.8	4.1 @ 2 days
20 , NMM	0.08	0.46 @ 2 days

N-methyl **16** in 1 h. Replacement of NMM with DBU increased the amount of methylation in 1 h to 1.8%. Surprisingly, the methyl group is not derived from NMM.

With NMM eliminated as the source, CDMT and DMF remained. ¹³C₂ and D₆ labeled CDMT **19** (Figure 4) was

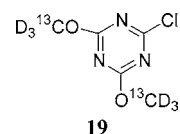


Figure 4.

(7) Kunishima, M.; Kawachi, C.; Morita, J.; Terao, K.; Iwasaki, F.; Tani, S. *Tetrahedron* **1999**, 55, 13159–13170.

(8) Upon review, use of *N*-ethylmorpholine instead of DBU was suggested.

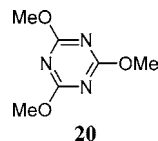


Figure 5.

prepared.⁹ Reaction under the standard conditions for 1 h showed complete incorporation of the labels by HPLC/MS into the *N*-methyl impurity **16**.

This result led to more questions that needed to be addressed. Clearly the methylating species is related to CDMT; several possibilities remain. The source could be the excess CDMT, the active ester **7**, the byproduct(s) of CDMT after the peptide coupling, or perhaps an impurity common to all samples of CDMT (including the labeled **19**). To completely ensure the appropriateness of our control strategy, this level of understanding is required. In particular, if the true methylating agent was an impurity in CDMT, a specification on the impurity would be established. We consider it likely that all sources of CDMT, including **19**, are prepared from cyanuric chloride and methanol, so common impurities are possible.

Therefore, determining if an impurity in CDMT was the actual problem was of primary importance. HPLC/MS of the lot of CDMT used for these studies revealed (by both UV and total ion count) only one impurity above the noise level, 2,4,6-trimethoxy-1,3,5-triazine **20** (Figure 5).

A full equivalent of **20**¹⁰ was tested under the more strenuous (100 °C) conditions and found to produce minimal **16** after an hour (Table 2). To provide some assurance that an impurity was not missed, perhaps due to decomposition under the HPLC conditions, a sample of CDMT from a second source (Aldrich) was tested and found to be indistinguishable (Table 1).

It was next determined if the byproduct(s) of CDMT from the peptide coupling was the methylating reagent. This is answered by the experiment under the strenuous conditions, employing isolated **10** as the substrate. Under these conditions CDMT byproducts could not be formed, yet the control reaction showed substantial *N*-methyl **16** formation in an hour. Therefore, CDMT byproducts from the peptide coupling are not required for methylation.

Given that the CDMT*NMM complex is the methylating agent, understanding the dependence on stoichiometry becomes important. Surprisingly, doubling the charges of CDMT and NMM (to 2.7 equiv) produced no significant change in the level of *N*-methyl **16** when sampled after 1 h. While this constitutes a great result for one designing a control strategy, it was difficult at first to understand. It is now believed that a combination of the poor solubility of the CDMT*NMM complex and the instability of the complex are responsible for this lack of sensitivity to stoichiometry. When NMM is added to a DMF solution of CDMT at

ambient, a precipitate forms immediately. Removal of an aliquot after 30 s, filtration, and HPLC analysis shows a barely detectable (below the quantitation limit) level of CDMT in solution. When sampled after 30 min, all that is observed in solution is the decomposition product **18** (eq 1). After 20 h the yield of **18** is near quantitative.

Only one set of data remains to be understood at this point: Tables 1 and 2 reveal that on prolonged exposure further *N*-methylation occurs, long after the CDMT*NMM complex has decomposed. At least one other species must be capable of *N*-methylation at a very slow rate. The slower *N*-methylation is sensitive to stoichiometry. With 1.1 equiv of CDMT, no further methylation was observed after 20 h. This suggests that decomposition of the excess CDMT*NMM produces the weaker methylating agent. The labeling experiment was only conducted for 1 h, so either **18** or the sideproduct containing the methyl originally on NMM (presumably methyl chloride) could be the second agent. As these prolonged reaction times are irrelevant to the manufacturing process, and the identity of the second methylating agent would not impact the control strategy, the identity of the second agent was not determined.

It is believed at this point that sufficient understanding has been gained to ensure long-term control of *N*-methylation. The CDMT*NMM complex is formed at 0 °C in the presence of **6**. Solubility of the complex is very low but sufficient to allow the very fast reaction with **6** to form the active ester **7**. The excess of CDMT*NMM is protected from decomposition by insolubility and temperature. *N*-methylation of **6** or **7** does not occur at 0 °C during the time scale of the process. L-GADE is added, and the mixture is warmed. As the mixture warms, the excess CDMT*NMM gains solubility and *N*-methylates competitively with decomposition. If held for excessive time, a decomposition product is also capable of *N*-methylation.

Based on this understanding of the mechanism of *N*-methylation, it is concluded that the current control strategy is adequate. As long as the CDMT stoichiometry, specified times, and temperatures during the peptide coupling are maintained, the level of *N*-methyl impurity will not increase. Even a massive excess of CDMT has no impact unless times are extended or temperatures are dramatically increased. Variation in quality of CDMT will have no impact, as CDMT itself is the active agent.

Experimental Section

Isolation and Characterization of *N*-Methyl Pemetrexed (12**).** *N*-Methyl Pemetrexed (**12**) was isolated from a production batch of Pemetrexed Disodium Heptahydrate (**1**) by preparative reversed-phase HPLC. Repeated injections of a 100 mg/mL solution of **1** in water were needed. Chromatography was conducted on a Kromasil C18 (50 mm × 250 mm, 10 μm) column. Separation was achieved using ammonium formate buffer (pH 3.5) and a gradient ramp from 12 to 25% acetonitrile over 25 min. **12** was isolated from the combined fractions by rotary evaporation, followed by lyophilization.

HRMS found (*M* + 1) 442.1704, calculated for C₂₁H₂₃N₅O₆ 441.1648 (calculated for *M* + 1 442.1726). All NMR spectra

(9) Prepared by use of appropriately labeled methanol in the procedure described in: Cronin, J. S.; Ginah, F. O.; Murray, A. R.; Copp, J. D. *Synth. Commun.* **1996**, *26*, 3491–3494.

(10) Obtained from Aldrich.

(11) Willker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. *Magn. Reson. Chem.* **1993**, *31*, 287–292.

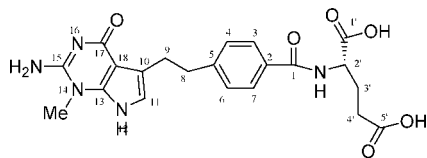


Figure 6. Numbering scheme for NMR assignments in Table 3.

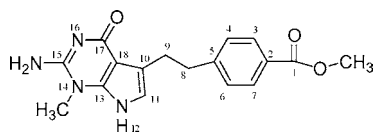


Figure 7. Numbering scheme for NMR assignments in Table 4.

Table 3. NMR assignments for *N*-methyl Pemetrexed (**12**)

atom number	type	¹ H ppm	¹³ C ppm	confirmation
1	>C=O	n/a	166.22	¹³ C shift, HMBC ¹¹
2	>C=	n/a	145.94	¹³ C shift, HMBC
3	arylCH	7.8	127.33	¹ H shift, ¹³ C shift, HSQC ¹¹ , d
4	arylCH	7.3	128.21	¹ H shift, ¹³ C shift, HSQC, d
5	>C=	n/a	131.57	¹³ C shift, HMBC
6	arylCH	7.3	128.21	¹ H shift, ¹³ C shift, HSQC, d
7	arylCH	7.8	127.33	¹ H shift, ¹³ C shift, HSQC, d
8	CH ₂	2.98	36.12	¹ H shift, ¹³ C shift, HSQC, m
9	CH ₂	2.9	27.52	¹ H shift, ¹³ C shift, HSQC, m
10	>C=	n/a	119.61	¹³ C shift, HMBC
11	arylCH	6.49	113.58	¹ H shift, ¹³ C shift, HSQC, s
12	NH	not observed	n/a	HMBC
13	>C=	n/a	139.25	¹³ C shift, HMBC
14	N	n/a	n/a	
14-CH ₃	CH ₃	3.55	32.06	¹ H shift, ¹³ C shift, HSQC, s
15	>C=	n/a	152.44	¹³ C shift, HMBC
16	N	n/a	n/a	
17	>C=O	n/a	164.17	¹³ C shift, HMBC
18	>C=	n/a	101.19	¹² C shift, HMBC
1'	>C=O	n/a	174.2	¹³ C shift, HMBC ^a
2'	CH ₁	4.36	52.25	¹ H shift, ¹³ C shift, HSQC, m
3'	CH ₂	2.02, 1.92	26.44	¹ H shift, ¹³ C shift, HSQC, m
4'	CH ₂	2.3	30.83	¹ H shift, ¹³ C shift, HSQC, m
5'	>C=O	n/a	174.04	¹³ C shift, HMBC ^a

^a Insufficient resolution to distinguish.

were collected on an Avance 300 spectrometer equipped with a 5 mm broad-band inverse (BBI) detection probe, with the exception of the Survey ¹³C spectrum which was collected with a QNP probe. All spectra were collected in DMSO-*d*₆. Assignments are made against the numbering scheme shown in Figure 6. Spectra, assignments, and confirming experiments are summarized in Table 3.

Independent Synthesis of *N*-Methyl Pemetrexed (12**, LSN2071949).** Methyl iodide (0.75 mL, 12.1 mmol) was added to a stirred slurry of Pemetrexed Disodium Heptahydrate **1** (2.0 g, 3.35 mmol) and triethylamine (0.75 mL, 5.38 mmol) in DMF (25 mL) at 24 °C under a nitrogen atmosphere. The resulting slurry was stirred at 24 °C for 3

Table 4. NMR assignments for **14**

atom number	type	¹ H ppm	¹³ C ppm	confirmation
1	>C=O	n/a	166.2	HMBC
1-OCH ₃	CH ₃	3.82	51.75	¹ H shift, HSQC, HMBC
2	>C=	n/a	127.13	HMBC
3	arylCH	7.87	138.85	¹ H shift, HSQC, HMBC d
4	arylCH	7.36	128.47	¹ H shift, HSQC, HMBC d
5	>C=	n/a	148.05	HMBC
6	arylCH	7.36	128.47	¹ H shift, HSQC, HMBC d
7	arylCH	7.87	128.85	¹ H shift, HSQC, HMBC d
8	CH ₂	2.98	35.88	¹ H shift, HSQC, HMBC m
9	CH ₂	2.92	26.88	¹ H shift, HSQC, HMBC m
10	>C=	n/a	119.63	HMBC
11	arylCH	6.61	114.46	¹ H shift, HSQC, HMBC s
12	NH	not observed	n/a	
13	>C=	n/a	139.61	HMBC
14	N	n/a	n/a	
14-NCH ₃	CH ₃	3.52	32.18	¹ H shift, HSQC, HMBC s
15	>C=	n/a	152.38	HMBC
16	N	n/a	n/a	
17	>C=O	n/a	163.16	HMBC
18	>C=	n/a	100.54	HMBC

h. Additional methyl iodide (0.2 mL, 3.2 mmol) and triethylamine (0.2 mL, 1.4 mmol) were added and the resulting solution was allowed to stir at 24 °C for 17 h. Additional methyl iodide (0.2 mL, 3.2 mmol) and triethylamine (0.2 mL, 1.4 mmol) were added, and the resulting solution was stirred at 24 °C for 20 h. At this point HPLC analysis showed no further reaction occurred from the last addition. Water (40 mL) was added to the reaction solution and extracted with dichloromethane (3 × 40 mL). The organic layer was washed with saturated brine (50 mL), dried (Na₂SO₄), filtered, and concentrated to an oil. Sodium hydroxide (1 N, 13.4 mL, 13.4 mmol) was added to the oil at 24 °C under nitrogen, and the resulting slurry was stirred for 30 min. To the resulting solution, water (13.4 mL) and ethanol (13.4 mL) were added. The pH of the solution was adjusted to 3.5 with HCl (3.2 N). The resulting slurry was heated to 65 °C, and dissolution occurred. The hot solution was allowed to cool to 24 °C. The resulting slurry was stirred at 24 °C for 30 min and then at 0–5 °C for 60 min. The green slurry was filtered and dried (ambient pressure and 24 °C) to afford **12** (0.97 g, 65%). Preparative chromatography was used to prepare the analytical sample.

Preparation of *N*-Methyl Intermediates (14**, **16**).** Methodology similar to that described above for **12**, omitting the saponification step, was used to prepare **14**. Assignments are made against the numbering scheme shown below (Figure 7). Spectra, assignments, and confirming experiments are summarized in Table 4. A DMF solution of intermediate **9** enriched in **16** was prepared by treatment of **10** under similar conditions.

Acknowledgment

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