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Research Article

Uncatalyzed microwave deuterium exchange labeling of bleomycin A_2

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Summary

Bleomycin sulfate in D_2O was deuterated using microwave irradiation under catalyst free conditions. Following the removal of labile deuterium and purification, bleomycin A_2 with mass $M+1$ to $M+7$ was obtained. Successful selective uncatalyzed microwave deuterium exchange reactions on examples from the following classes of heterocycles are also described: imidazole, thiazole, indole, purine, and quinazoline. The described method was used as a test for non-labile active protons. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: microwave; deuterium; exchange labeling; bleomycin-[D_n]

Introduction

Bleomycins[1] are a group of glycopeptide antibiotics isolated from bacteria. Bleomycin A_2 is the main component employed clinically as an anti-tumor antibiotic. Stable isotope labeled bleomycin A_2 containing $> M+3$ isotope labeling with no detectable $M+0$ was needed as a mass spectroscopic standard for mouse stratification studies of bleomycin clearance using SNPs prediction. Though bleomycin-labeling methods[2,3] have been described in the literature, these methods require bacterial uptake of labeled unnatural amino acids or other sources of isotopes during fermentation. The bleomycins must then be extracted from the culture, and the desired-labeled bleomycin must be isolated and identified. In many cases the % isotope incorporation is low. Within the glycopeptide structure, bleomycins contain four nitrogen heterocycles, three of which appeared to be amenable to deuterium isotope exchange. One of the rings is an imidazole and the other two are conjoined thiazoles. Microwave induced deuterium isotope exchange on heterocycles using catalysts have appeared in the literature.[4,5] These reactions were catalyzed

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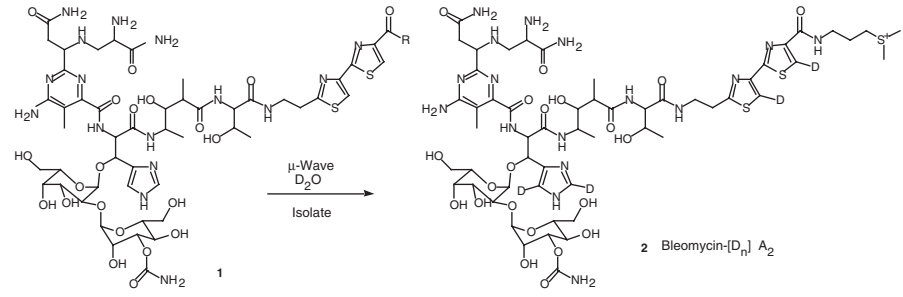


Bleomycin A_2 R = $(CH_3)_2S^+CH_2CH_2CH_2NH$-
Bleomycin B_2 R = $H2NC(NH)NHCH_2CH_2CH_2CH_2NH$-
Bleomycin Sulfate (~60% Bleomycin A_2 + ~30% Bleomycin B_2)

Scheme 1.

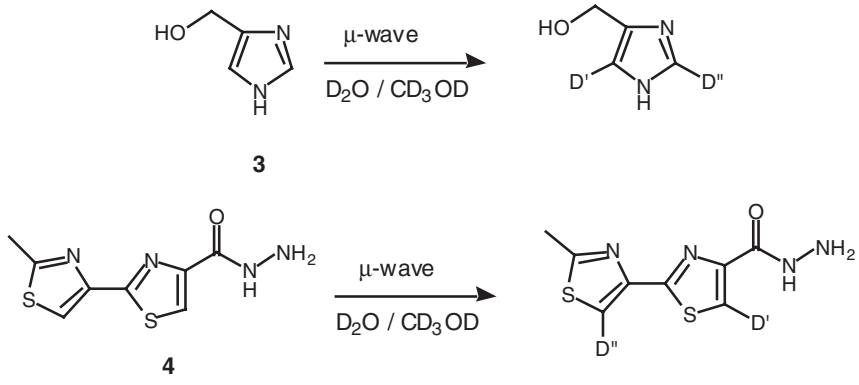
by acids, bases and metals such as Rh and Raney Ni. These methods offered a potentially much simpler method of incorporation of deuterium label directly into bleomycin (Scheme 1).

However, since the thiazole groups would likely be incompatible with the described catalysts, we decided to investigate the possibility of the catalyst free microwave induced labeling of bleomycin. To test the feasibility of this idea, we first attempted the catalyst free microwave induced deuterium exchange on commercially available model compounds 4-(hydroxymethyl) imidazole **3**, and 2-(2-methylthiazol-4-yl)thiazole-4-carboxylic acid hydrazide **4**. Compound **3** was used as a model for the imidazole moiety and compound **4** was used as a model for the thiazole moiety. Conditions were readily worked out for the full and specific incorporation of deuterium into both of the model compounds (Scheme 2).

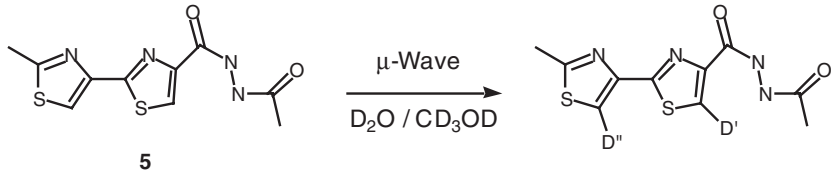
If the model reactions translated directly to bleomycin, an isotope incorporation of at least $M + 4$ was potentially attainable. Using the model reactions as a guide, we attempted to label bleomycin sulfate directly with deuterium by the same method. In the course of our experimentation with model compounds, it became apparent that uncatalyzed microwave induced deuterium exchange was highly selective. Thus, to extend this technique to other nitrogen heterocycles of interest to us, we tested the method on representative molecules from other classes. The cases described are those in which selective isotope incorporation was complete and no degradation occurred.

Results and discussion

Since this was our first encounter with a microwave apparatus designed specifically for chemical reaction use, we conducted a series of experiments to familiarize ourselves with the behavior of the apparatus. Some of these experiments are described below. Other approaches[5] for microwave exchange



Scheme 2.



Scheme 3.

reactions described in the literature use open flasks to avoid pressure build up. This necessitates low temperature reactions to avoid solvent boil-over and repeated cooling and replacement of evaporated solvent. Furthermore, with an open system, temperature regulation is difficult. The apparatus available to us is designed for up to 20 atm pressure and uniform temperature applications. Two types of heavy wall glass tubes are available. One for volumes of 0.5–2.0 ml and the other for 2–5 ml applications. Magnetic spin bars and metal crimped Teflon seals are supplied with the tubes. A recent review article[4] and references therein describes the basics of microwave applications and theory. Also, comparison with thermal reactions has appeared.[5] One of our first experiments was to convince ourselves of the feasibility of microwave irradiation compared to the thermal process for labeling model compound **4**. Microwave enhanced conditions in 30% D_2O/CD_3OD were found to di-deuterate compound **4** fully and selectively. This required 10-min irradiation at 165°C and 13-atm pressure. An identical reaction run in a similar sealed tube for 10 min at 165°C in an oil bath progressed to 10% incorporation of D′ and only 3% incorporation of D″. From this we expected at least an order of magnitude enhancement due to the microwave irradiation for this compound (Scheme 3).

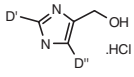
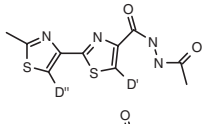
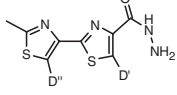
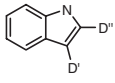
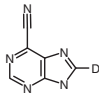
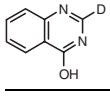
Interestingly, we found that the acetylated form **5** of the model compound **4** was resistant to microwave irradiation. It required 60 min of microwave

irradiation at 165°C to incorporate 60% D′ and 20% D″ in compound **5**. Since it has been reported that microwave isotope exchange is enhanced more by D_2O than CD_3OD solvents,[5] we hoped that bleomycin, which is soluble in D_2O, would be more readily exchanged in pure D_2O than the model compound, which required added CD_3OD for solubility (Table 1).

Since model deuterium exchange reactions were highly selective, we tested a few other nitrogen heterocycles of interest to us. The results of the successful experiments are listed in the table below. Experiments 1–5 show the order of deuterium exchange in the model compounds. Experiments 5 and 6 compare reactions in the presence and in the absence of microwave irradiation. Experiments 7–8 show the enhancing effect of DCl in the solvent mixture for deuterium incorporation into indole. Experiments 9–10 demonstrate selectivity of deuterium incorporation. Experiments 11 and 12 compare deuterium exchange in 6-cyanopurine and 4-hydroxyquinazoline.

Our initial experiments using bleomycin sulfate in D_2O were run under the same conditions as the model compound (microwave irradiation at 165°C) except at shorter incremental time intervals. Bleomycin sulfate **1** is a mixture of bleomycins containing mainly bleomycin A_2 (~60%) and bleomycin

Table 1. Microwave deuterium isotope exchange

Compound	Expt No.	Solvent	Temp (°C)	Pressure (atm)	Time (s)	%D Incorporated
	1	D2O	165	10	1000	D′ > 95, D″ = 20
	2	D2O	190	18	3600	D′ > 95, D″ > 95
	3	30% D2O/CD3OD	165	13	3600	D′ = 60, D″ = 20
	4	30% D2O/CD3OD	165	13	300	D′ > 95, D″ = 50
	5	30% D2O/CD3OD	165	13	600	D′ > 95, D″ > 95
	6[a]	30% D2O/CD3OD	165	13	600	D′ = 10, D″ = 3
	7	CD3OD	165	15	3600	D′ = 50, D″ = 0
	8	CD3OD + DCl	165	15	3600	D′ > 95, D″ = 30
	9	50% D2O/CD3OD	165	13	1000	D′ > 95, D″ = 0
	10	50% D2O/CD3OD	165	13	3600	D′ > 95, D″ = 0
	11	D2O	165	10	3600	> 95
	12	50% D2O/CD3OD	165	12	300	> 95

[a] This reaction was performed at 165°C in an oil bath.

B_2 ($\sim$30%). Since bleomycins are glycopeptides, they are expected to undergo degradation under prolonged microwave irradiation. Initial experiments were set up to compare deuterium isotope incorporation versus degradation of bleomycin sulfate at the temperature dictated by the model compounds at increasing time intervals. Isotope incorporation could be followed by NMR analyses directly in the D_2O reaction mixture. Once it was found that sufficient isotope incorporation occurred for our purposes, conditions were optimized to prepare isotopically labeled Bleomycin A_2. These reactions were followed by LC/MS analysis of reaction aliquots in which labile deuterium had been removed by proton exchange. This initial experiment is tabulated below (Table 2) and the details are described in the Experimental Section.

The area norm (AN%) column shows the area-normalized % of bleomycin A_2 remaining after each subjection to microwave radiation at 165°C for 2 min intervals. The remaining columns show the incremental increase of molecular weight because of deuterium incorporation. For example, the T_{10} sample shows that about 15% of the reaction mixture is bleomycin A_2 and 21.5% of the compound is MW $M + 5$, with no measurable interference from $M + 0$. The parent compound T_0 contains significant $M + 1$ to $M + 3$ and measurable $M + 4$, thus a shift is observed up to $M + 8$ in some cases (not shown here). Since the original mixture contained 61% bleomycin A_2, the adjusted LC/MS yield is about 25%. A similar experiment was run at 150°C at 5 min intervals to test if lower temperature for a longer time would improve the results. The ratio of deuteration to degradation decreased at the lower temperature. Temperatures above 165°C were avoided since the shorter reaction times would be difficult to replicate because heating and cooling times are inconsistent from sample to sample. Since we also found that the deuteration to degradation ratio decreased as we scaled up the reaction from 2 to 15 mg of

Table 2. Bleomycin sulfate $\xrightarrow[D_2O]{\mu\text{-Wave}}$ bleomycin-D(n) sulfate

		Isotope distribution (percent peak area of extracted ions)						
		m/z 1414	m/z 1415	m/z 1416	m/z 1417	m/z 1418	m/z 1419	m/z 1420
Sample	AN%[a] (UV)	0D	1D	2D	3D	4D	5D	6D
T_0	61.4	40.7	33.7	14.6	8.8	2.2		
T_2	38.5		28.4	29.6	23.1	12.2	6.2	0.7
T_4	27.3		13.1	30.7	28.5	16.6	8.5	2.7
T_6	22.1		6.6	25.1	29.2	19.2	12.6	6.6
T_8	18.6		1.0	20.6	29.4	20.0	16.4	9.0
T_{10}	15.6			14.6	27.8	23.8	21.5	12.2

[a] AN% is the area-normalized % of bleomycin A_2 remaining after each microwave irradiation at 165°C for 2 min.

bleomycin sulfate even at reduced concentrations, larger scale reactions (>15 mg) were not attempted. Instead, we ran consecutive runs at an optimized 6 min each at 165°C and combined them for work up.

Experimental

Microwave irradiation was performed using a *Smith Creator* (by Personal Chemistry) microwave apparatus using D_2O (Aldrich, 99.9 atom % D) and CD_3OD (Aldrich, 99.8+ atom % D) and 35% DCl in D_2O (Sigma, 99 atom % D). Proton spectra were run on a Bruker 300 MHz NMR spectrometer. LC/MS data were recorded from an Agilent 1100 Series LC/MSD. TLC plates (silica gel 250 μm) were obtained from Analtech. Bleomycin Sulfate was obtained from LKT Laboratories. The model compound 4-(hydroxymethyl)imidazole hydrochloride 98% was obtained from Aldrich and 2-(2-methylthiazol-4-yl)thiazole-4-carboxylic acid hydrazide was obtained from Maybridge. The other small molecules were reagent grade, used as purchased from various sources.

Typical small molecule experiment

The substrate (20 mg) was weighed into a standard 0.5–2.0 ml capacity conical heavy walled microwave tube containing a triangular micro spin-bar. Following preliminary solubility testing, the substrate was dissolved in a minimum of CD_3OD (0.0–1.8 ml) and diluted with D_2O to give a total volume of 2.0 ml of clear solution. An approximate 0.5 ml aliquot was removed as control. The tube was sealed (metal crimped Teflon lined septum), inserted into the pressure sealed slot, and subjected to microwave irradiation with stirring. After 300 s (5 min) at the desired temperature the tube was cooled, and an approximate 0.5 ml aliquot was transferred to a NMR tube. The D-exchange reaction was judged by the disappearance of H peak intensity (i.e. replacement with D) in the proton-NMR spectrum vs the control. The remainder of the reaction volume was re-sealed and subjected to further microwave irradiation as appropriate. Apparent successful reactions were confirmed by first exchanging labile protons by concentrating the NMR solution twice from 5 ml each MeOH, re-dissolving in MeOH, and analyzing by MS vs the control sample.

Typical bleomycin optimization experiment

The bleomycin sulfate (2 mg) was dissolved in 2.0 ml D_2O. A 0.2 ml T_0 sample was removed, and the remainder was subjected to microwave irradiation, maintaining a temperature of 165°C, and a pressure of 10 atm for 2 min, the reaction was cooled and a 0.2 ml T_2 sample was removed. This procedure was repeated for each increment to T_{10}. In initial runs, the NMR spectrum was run

directly on the reaction mixture to follow the hydrogen–deuterium exchange in the imidazole and thiazole moieties. Each sample was concentrated twice from methanol to remove exchangeable deuterium, and examined by LC/MS analysis.

Typical bleomycin isolation experiment

Bleomycin sulfate (26 mg) was dissolved in 5.2 ml D_2O. Then, 2.6 ml portions of the clear colorless solution were transferred to separate heavy-walled 5 cc microwave tubes, each containing a spin-bar. Both tubes were individually sealed using a metal crimped Teflon lined septum, and each was successively submitted to microwave irradiation at a preset temperature of 165°C for 6 min (at 10 atm.). After cooling to ambient temperature, the clear, nearly colorless contents of the tubes were combined and the solutions were concentrated to dryness three times from 20 ml portions of methanol, to exchange labile deuterium. The residual white solid was redissolved in 0.5 ml water, and then spotted onto four 10×20 cm 250 μ silica gel TLC plates. The plates were eluted with (2:3) 1% aqueous ammonium formate/methanol.[6] The major most polar band (R_f 0.2) was scraped, slurried with water to remove salts, and then slurried $4\times$ with eluent by repeated vortex, centrifuge, and decantation. LC/MS analysis of this product in water indicated about 35% (1.5 mg) Bleomycin-[D_n] A_2 (deuteration: 0% $M+0$, 8% $M+1$, 21% $M+2$, 27% $M+3$, 22% $M+4$, 13% $M+5$, 6% $M+6$, and 3% $M+7$). The bulk of the impurities had molecular weights at least 98 mass units less than bleomycin. Further purification of about 1/3 of the partially purified product by preparative HPLC techniques (C18, 9.4/250, (12:88) ACN: 0.1% TFA) afforded 0.16 mg of 56% pure bleomycin-[D_n] with a similar isotope distribution. Additional purification was not required because the impurities did not interfere with the molecular ion of the parent labeled compound.

Conclusion

We have developed an abbreviated strategy for the direct, catalyst free, microwave induced deuterium labeling of bleomycin sulfate in D_2O, as established from model compounds. Following the removal of labile deuterium and purification, bleomycin A_2 with mass $M+1$ to $M+7$ was obtained containing no detectable $M+0$. Successful selective uncatalyzed microwave deuterium exchange reactions on examples from the following classes of heterocycles were also described: imidazole, thiazole, indole, purine, and quinazoline. The described method may be used as a test for non-labile active protons.

Acknowledgements

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