MULTI-^{13}C-LABELLED INHIBITORS OF TUBULIN ASSEMBLY: 5-SUBSTITUTED METHYL N-(1H-BENZIMIDAZOL-2-YL)CARBAMATES

H.T.Andrew Cheung[+] Diem Dieu Chau[+] and Ernest Lacey[‡]

[+]Department of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia

[‡]McMaster Laboratory, Division of Animal Health, CSIRO, Private Bag No.1, P.O. Glebe, NSW 2037, Australia

SUMMARY

Eight methyl N-(1H-benzimidazol-2-yl)carbamates with various 5-substituents were synthesized, each ^{13}C-enriched at carbon 2, and the carbonyl and methoxy carbons. Five were prepared by cyclization involving the appropriate 4-substituted 1,2-diaminobenzene (C_6H_5CO-, $CH_3CH_2CH_2O$-, $CH_3CH_2CH_2S$-, C_6H_5S- and $CH_3CH_2CH_2CH_2$-), and methyl-^{13}C N-[imino(methylthio)methyl-^{13}C]carbamate-^{13}C or methyl N,N'-bis(methoxy-^{13}C-carbonyl-^{13}C)carbamimidothionate-^{13}C. The latter were prepared from commercially available ^{13}C-enriched (91-92 atom %) carbon tetrachloride, methanol, and thiourea. The remaining three (5-substituents: $C_6H_5CH(OH)$-, C_6H_5SO- and $CH_3CH_2CH_2SO$-) were prepared by side-chain reduction or oxidation. The ^{1}H- and ^{13}C-NMR, and the methane CI mass spectral data of the products and intermediates are presented.

Key words: anthelmintic, microtubules, ^{13}C-enriched, methyl chloroformate, S-methyl-isothiourea, ^{13}C-NMR.

INTRODUCTION

Of importance in cellular processes is the assembly of α- and β-subunits of the protein tubulin into tube-like

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polymers called microtubules which perform a variety of vital functions in the cell. Certain compounds such as colchicine and the anticancer drugs vincristine and vinblastine bind to tubulin, causing inhibition of tubulin assembly, thus disrupting the viability of microtubules, with resultant cell death.[1] In the special case of the benzimidazole carbamate group of anthelmintic drugs used widely to control nematode parasites in sheep, correlation exists between anthelmintic potency and ability to bind tubulin.[2,3] Furthermore, developed drug resistance has been shown to be associated with an alteration in the molecular structure of tubulin, resulting in reduction of binding.[4,5]

Recent years have witnessed elegant uses of ^{13}C-enriched molecules as sensitive probes in ^{13}C-NMR studies of enzyme - small molecule interactions.[6] We have undertaken an NMR study of the interaction between pure tubulin from resistant and susceptible nematodes[5] and benzimidazole carbamates, and report here the syntheses of eight 5-substituted methyl N-(1H-benzimidazol-2-yl)-carbamates (1a) - (1h) each with ^{13}C-enrichment at carbon 2, and the carbonyl and methoxy carbons (Figure 1). The three labelled positions have been chosen as they are known to be at or near the site involved in binding to tubulin.[3] The choice of 5-substituents was based on the wide range of tubulin binding activity and resistance ratios exhibited by methyl N-(1H-benzimidazol-2-yl)carbamates with these substituents.[2,3,5]

RESULTS AND DISCUSSION

5-Substituted alkyl N-(1H-benzimidazol-2-yl)-carbamates are conveniently prepared either by reaction of a 5-substituted 2-aminobenzimidazole with an alkyl

haloformate[7] (cf. Figure 1, dotted arrows), or of a 4-substituted 1,2-diaminobenzene with an alkyl N-[imino-(methylthio)methyl]carbamate[8] (full arrows). We prefer the latter route whereby one single intermediate with the required array of three ^{13}C-enriched carbons, viz. methyl-^{13}C N-[imino(methylthio)methyl-^{13}C]carbamate-^{13}C (6), serves to yield various 5-substituted methyl N-(1H-benzimidazol-2-yl)carbamates with the appropriate ^{13}C-labels. In practice, this intermediate formed from S-methyl(isothiourea-^{13}C) (3) and methyl-^{13}C chloroformate-^{13}C (4) under basic conditions was usually accompanied by methyl N,N'-bis(methoxy-^{13}C-carbonyl-^{13}C)carbamimidothionate-^{13}C (7). In model experiments using unlabelled reactants in aqueous solution at pH 8, the ratio of the di- and mono-carbomethoxy intermediates [cf. (7) vs. (6)] as determined by ^{1}H-NMR was 1:2 when the two reactants were in equimolar amounts, and rose to 5:1 when methyl chloroformate was 50% in excess.* In syntheses using ^{13}C-enriched reactants, the former conditions were adopted to minimize the formation of the dicarbomethoxy intermediate (7), which was expected to form the required 5-substituted methyl N-(1H-benzimidazol-2-yl)carbamate (5)[9] with loss of one carbomethoxy group, and hence half of its enriched carbons. Intermediates (6) and (7) generated in situ [or in one case, isolated intermediate (6)], and buffered with acetic acid - sodium acetate were then treated with 4-benzoyl-, 4-propoxy-, 4-propylthio-, 4-phenylthio- and 4-n-butyl-1,2-diaminobenzene (2a) - (2e) to yield the corresponding 5-substituted methyl-^{13}C N-(1H-benzimidazol-

*Impurities were present when S-methylisothiourea was 50% in excess. Replacement of aqueous hydroxide by pyridine in toluene increased the amount of the dicarbomethoxy product.

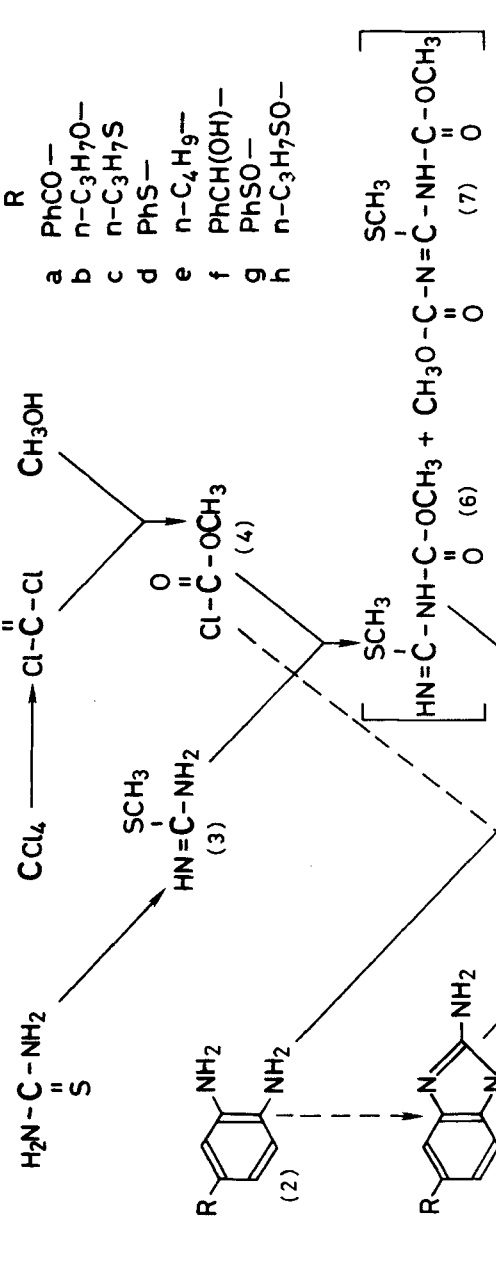

Figure 1. Synthetic scheme for 5-substituted methyl N-(1H-benzimidazol-2-yl) carbamates (1a-e). Enlarged C represents position of ^{13}C enrichment.

2-^{13}C-2-yl)carbamate-^{13}C (1a) - (1e) (Figure 1). The diamines were prepared according to published literature methods as cited in Table 1.

The remaining ^{13}C-labelled 5-substituted methyl N-(1H-benzimidazol-2-yl)carbamates (1f) - (1h) (see Table 1) were prepared by modification of the 5-side-chain. Sodium borohydride reduction of the benzoyl group of ^{13}C-labelled mebendazole (1a) gave the analog (1f) having a hydroxybenzyl side-chain, and corresponding to a major metabolic product of mebendazole in vitro.[10] Oxidation of the phenylthio and propylthio group of ^{13}C-labelled fenbendazole (1d) and albendazole (1c) respectively, using hydrogen peroxide in acetic acid, yielded the respective sulfoxide analogs (1g)(oxfendazole) and (1h), which also correspond to metabolites in animals.[11,12]

Intermediates S-methyl(isothiourea-^{13}C) sulfate (3) and methyl-^{13}C chloroformate-^{13}C were respectively prepared by methylation of thiourea-^{13}C with dimethyl sulfate,[13] and by reaction of methanol-^{13}C with phosgene-^{13}C, the latter generated from carbon-^{13}C tetrachloride.[14]

TABLE 1

Mass spectrometric and melting point data of 5-substituted methyl-^{13}C N-(1H-benzimidazol-2-^{13}C-2-yl)carbamate-^{13}C

Compounds	Trivial name of unlabelled analog	Mass spectral data[a] MH^+	other ions	m.p.°C	Lit. m.p. (unlabelled)[b]
(a) By cyclization (Figure 1)[c]					
(1a)	mebendazole	299	221[d]	286-290°	288.5°(ref.9)
(1b)	oxibendazole	253	211[e]	200-202°	230-230.5°(ref.17)
(1c)	albendazole	269		197-200°	208-210°(ref.18)
(1d)	fenbendazole	303	225[d]	218-222°	243°dec.(ref.15)
(1e)	parbendazole	251	207	193-198°	218-222°(ref.7)
(b) By side-chain modification					
(1f)	reduced mebendazole	301	283,223,205[f]	>310°	
(1g)	oxfendazole	319		284-287°	ca.253°; ca. 275-278°(ref.15)
(1h)	albendazole sulfoxide	285		>310°	

a Chemical ionisation was achieved using methane as the reagent gas and therefore MH^+ ions were observed with associated adduct ions at M+29 and M+41 ($C_2H_5^+$ and $C_3H_5^+$ respectively)

b Due to extensive reports of polymorphism within this class of substances (cf. ref. 15), melting point comparisons are not too meaningful

c The starting diamine (2a) was obtained commercially (Aldrich Chemicals) while (2b) - (2e) were prepared according to references 2, 12, 15 and 16 respectively

d $MH^+ - C_6H_6$ e $MH^+ - C_3H_6$ f $MH^+ - H_2O$ and/or C_6H_6

TABLE 2

Spectroscopic data of ^{13}C-enriched intermediates

Compounds	^{13}C-NMR data[a]			^{1}H-NMR data[c]	
	$O\underline{C}H_3$	$\underline{C}=O$	$HN=\underline{C}-SCH_3$	OCH_3	SCH_3
$CH_3O\underline{C}OCl$ (4)	57.9 dd[b] ($^{1}J_{CH}$ 149, $^{2}J_{COC}$ 2.8)	151.2 m	-	3.90 dd ($^{1}J_{CH}$ 150, $^{3}J_{COCH}$ 4.7)	-
$CH_3O\underline{C}ONH\underline{C}(SCH_3)=NH$ (6)	52.8	162.6	173.9	3.74 dd ($^{1}J_{CH}$ 146, $^{3}J_{COCH}$ 3.9)	2.47 d ($^{3}J_{CSCH}$ 4.6)
$[H_2N\underline{C}(SCH_3)=NH_2^+]_2\ SO_4^{--}$ (3)	-	-	173.5[d]	-	

a Chemical shifts of ^{13}C-enriched carbons (shown thickened) in p.p.m. (and coupling constants in Hz) measured (unless otherwise stated) in chloroform-^{2}H with δ_C ($CDCl_3$) = 77.0

b For gated decoupling conditions see footnote a of Table 3

c Chemical shifts in p.p.m. from tetramethylsilane (and coupling constants in Hz) measured in chloroform-^{2}H at 89.6 MHz

d In D_2O with dioxane (δ 67.4) as internal standard

TABLE 3

Nuclear magnetic resonance spectroscopic data of 5-substituted methyl-^{13}C N-(1H-benzimidazol-2-^{13}C-2-yl)carbamate-^{13}C

Compounds	Gated ^{13}C-NMR data[a]			^{1}H-NMR data[b]				
	OCH_3	C-2	NHCOO	OCH_3	H-6	H-7	H-4	Other protons
(1a)	52.6 q ($^{1}J_{CH}$ 147.5)	149.4	154.5	3.77 dd ($^{1}J_{CH}$ 147.5, $^{3}J_{COCH}$ 4.0)	← 7.4 - 7.9 →			
(1b)	52.3 q ($^{1}J_{CH}$ 147)	147.3	155.2[c]	3.73 dd ($^{1}J_{CH}$ 147, $^{3}J_{COCH}$ 4.1)	6.68 dd ($J_{6,7}$ 8.5, $J_{4,6}$ 2)	7.25 d ($J_{6,7}$ 8.5)	6.94 d ($J_{4,6}$ 2)	0.99 t(J 7) 1.69 m 3.89 t(J 7)
(1c)	52.4 q ($^{1}J_{CH}$ 147.5)	147.8	154.8	3.75 dd ($^{1}J_{CH}$ 147, $^{3}J_{COCH}$ 4.1)	7.09 dd ($J_{6,7}$ ca.8, $J_{4,6}$ ca.2)[d]	7.34 d ($J_{6,7}$ ca.8)[d]	7.42 d ($J_{4,6}$ ca.2)	0.95 t(J 7) 1.58 m 2.85 t(J 7)
(1d)	52.5 q ($^{1}J_{CH}$ 147.5)	148.2	154.6[c]	3.76 dd ($^{1}J_{CH}$ 147.5, $^{3}J_{COCH}$ 4.0)	← 7.1 - 7.5 →			
(1e)	52.3 q ($^{1}J_{CH}$ 147.5)	147.7	159.1	3.72 dd ($^{1}J_{CH}$ 147.5, $^{3}J_{COCH}$ 3.8)	6.96 dd ($J_{6,7}$ ca.8 $J_{4,6}$ ca.2)[d]	7.35 d ($J_{6,7}$ ca.8)[d]	7.26 d ($J_{4,6}$ ca.2)	ca.0.9[e] 1.1-1.7 m

(1f)	52.3 q ($^{1}J_{CH}$ 147)	147.9	155.4	3.72 dd ($^{1}J_{CH}$ 147, $^{3}J_{COCH}$ 4.0)	7.06 dd ($J_{6,7}$ 8.3, $J_{4,6}$ ca. 2)	← 7.2 - 7.4 →	5.85 q (J 4)
(1g)	52.6 q ($^{1}J_{CH}$ 147.5)	148.8	154.3	3.76 dd ($^{1}J_{CH}$ 147.5, $^{3}J_{COCH}$ 4.0)	7.34 dd ($J_{6,7}$ 8.3, $J_{4,6}$ 1.6)	← 7.4 - 7.8 →	
(1h)	52.6 q ($^{1}J_{CH}$ 147.5)	148.6	154.5	3.77 dd ($^{1}J_{CH}$ 147.5, $^{3}J_{COCH}$ 4.1)	7.32 dd ($J_{6,7}$ 8.3, $J_{4,6}$ 1.8)	← 7.5 - 7.9 →	0.95[e] 1.53 m 2.80[e]

a Chemical shifts of ^{13}C-enriched carbons in p.p.m. (and ^{13}C-^{1}H coupling constants in Hz) measured at 22.5 MHz in dimethyl sulfoxide-$^{2}H_6$ under gated decoupling conditions (with broadband decoupler on during pulse delay of about 2 s), with $\delta(CD_3SOCD_3)$ = 39.5 p.p.m.

b Chemical shifts in p.p.m. from tetramethylsilane (and coupling constants in Hz) measured at 89.6 MHz in dimethyl sulfoxide-$^{2}H_6$, except for compound (1e) which was in 1:5 dimethyl sulfoxide-$^{2}H_6$/chloroform-^{2}H

c After removal of ^{13}C-^{1}H coupling by broadband ^{1}H decoupling, width-at-half-height changed by ca. 7 Hz due to removal of $^{3}J_{COCH}$ (cf. ^{1}H-NMR)

d Approximate first order analysis

e Distorted triplet

EXPERIMENTAL

All experiments with ^{13}C-enriched reactants were preceded by those involving unlabelled materials, often carried out repeatedly. Yields of unlabelled products are given only if higher than in the syntheses of the corresponding ^{13}C-labelled products. The spectral data of each synthesized 5-substituted methyl-^{13}C N-(1H-benzimidazol-2-^{13}C-2-yl)carbamate-^{13}C are given in Table 3, and those of intermediates in Table 2. Evaporation of solvents took place under reduced pressure at the lowest possible temperature. Melting points were obtained using a Kofler hot-stage apparatus and were uncorrected. ^{13}C-Enriched starting materials (91-92 atom %) were purchased from Amersham, U.K. (carbon-^{13}C tetrachloride and methanol-^{13}C) and from MSD Isotopes, U.S.A. (thiourea-^{13}C). ^{1}H- and ^{13}C-NMR data were collected using a JEOL FX-90Q spectrometer operating in the Fourier-transform mode at 89.6 MHz and 22.5 MHz respectively. Acquisition time, pulse delay and pulse width were, for ^{1}H: 4.6 s, 0.1 s, and 43 μs; for ^{13}C: 0.73 s, 0.1 s and 7 μs respectively. Chemical ionization mass spectra were obtained using a Finnigan 3200E GC - mass spectrometer and associated Finnigan 6110 data system.

Methyl-^{13}C chloroformate-^{13}C (4)

100% Sulfuric acid (formed by addition of fuming sulfuric acid to 98% sulfuric acid until yellow) (0.66 g, 6.7 mmol, or 55% excess) and freshly ignited celite (15 mg) were placed in a flask fitted with a dropping funnel and a delivery tube above a short reflux condenser. Carbon-^{13}C tetrachloride (1.0 g, 6.5 mmol) was added and the mixture was heated to 135-140°C. Phosgene-^{13}C formed was collected in dry toluene (2.3 g) in an ice-salt bath while hydrogen

chloride was allowed to exit via a calcium chloride drying tube. The increase in weight of the toluene solution after 2 h was 0.60-0.65 g. To the ice-cold solution of phosgene-^{13}C in toluene in a sealed vessel was injected methanol-^{13}C (167 mg, 5.1 mmol). The mixture was gently shaken and allowed to rise to room temperature after 30 min. After 2 h, ^{1}H-NMR analysis showed the presence of 3.6 mmol of methyl-^{13}C chloroformate (NMR data: Table 3), and 0.2 mmol of dimethyl-^{13}C carbonate-^{13}C, δ ($CDCl_3$) 3.76 dd ($^{1}J_{CH}$ 150, $^{3}J_{COCH}$ 4.1 Hz).

S-Methyl(isothiourea-^{13}C) sulfate (3)

To a stirred suspension of thiourea-^{13}C (0.50 g, 6.5 mmol) in water (0.4 ml) was added dimethyl sulfate (0.45 g, 3.6 mmol, 10% excess)(purified by being washed with 1 vol. ice-cold water followed by 1/3 vol. cold saturated aqueous sodium bicarbonate). The reaction was allowed to progress spontaneously under reflux and with mild cooling for 10 min. It was then refluxed for 1 h. To the cooled mixture 95% ethanol (2 ml) was added with stirring. The crystals formed were filtered, washed with cold ethanol (3 x 1 ml), and dried at room temperature under reduced pressure, yielding S-methyl(isothiourea-^{13}C) sulfate (3) (0.56 g, 62%) (yield of unlabelled analog 75%), m.p. 233-236°C (lit. [13] m.p. of unlabelled analog, 235°C).

Methyl-^{13}C N-[imino(methylthio)methyl-^{13}C]carbamate-^{13}C (6) and methyl N,N'-bis(methoxy-^{13}C-carbonyl-^{13}C)carbamimidothionate-^{13}C (7)

A stirred mixture of S-methyl(isothiourea-^{13}C) sulfate (3) (78 mg, 0.56 mmol) in water (0.4 ml) and of methyl-^{13}C chloroformate-^{13}C (4) (54 mg, 0.56 mmol) in toluene (0.44 g)

maintained at 5°C was brought to pH 8 by the addition of 4M aqueous sodium hydroxide (0.15 ml). The mixture was stirred vigorously at 5°C for 30 min and then allowed to warm to room temperature during 30 min, while the pH was maintained by the addition of sodium hydroxide. The product consisting of a mixture of the title compounds (6) (major) and (7) was used in situ for the next experiment. In one experiment at half scale the product was found to consist mainly of product (6); extraction into dichloromethane followed by evaporation of the solvent yielded methyl-^{13}C N-[imino(methylthio)methyl-^{13}C]carbamate-^{13}C (6) (23 mg, 54%), m.p. 69-70°C.

Syntheses of 5(6)-substituted methyl-^{13}C N-(1H-benzimidazol-2-^{13}C-2-yl)carbamates-^{13}C (1a)-(1e) from 4-substituted 1,2-diaminobenzenes (2a)-(2e)

The following general procedure was employed yielding the first four of the ^{13}C-labelled 5-substituted methyl N-(1H-benzimidazol-2-yl)carbamates shown in Table 1. The reaction mixture obtained by reacting equimolar quantities of S-methyl(isothiourea-^{13}C) sulfate (3) (0.56 mmol), and methyl-^{13}C chloroformate-^{13}C (4) at pH 8 as described in the previous paragraph was adjusted to pH 4-5 with 100% acetic acid. After successive addition of sodium acetate (23 mg, 0.28 mmol) and the appropriate 4-substituted 1,2-diaminobenzene (2a)-(2d) (0.28 mmol) as the free base or the hydrochloride salt, the mixture was stirred vigorously at 100°C for 90 min. The product precipitated from the cooled mixture was collected, triturated with cold methanol, filtered again, washed several times with cold methanol, and finally dried under vacuum. Yields were 94-96% based on the 4-substituted 1,2-diaminobenzene (2a)-(2d), and 47-48% based

on S-methyl(isothiourea-^{13}C) sulphate (3) and methyl-^{13}C chloroformate (4).

The fifth product shown in Table 1 was prepared in a similar manner, but starting from isolated methyl-^{13}C N-[imino(methylthio)methyl-^{13}C]carbamate-^{13}C (6) (22 mg, 0.145 mmol), and employing excess of the diamine (2e) (0.18 mmol); the yield was 30%.

Syntheses of 5(6)-substituted methyl-^{13}C N-(1H-benzimidazol-2-^{13}C-2-yl)carbamates-^{13}C (1f)-(1h) by side-chain modification

To a stirred suspension of ^{13}C-labelled methyl N-[5(6)-benzoyl-1H-benzimidazol-2-yl]carbamate (mebendazole) (1a) (20 mg, 0.067 mmol) in N,N-dimethylformamide (1.5 ml) and methanol (1.5 ml) was added sodium borohydride (21 mg, 0.55 mmol). After 45 min at room temperature when the reaction was completed as shown by TLC, traces of undissolved material was removed, and water (8 ml) was added to the filtrate. The precipitate formed on evaporation of the methanol was collected, washed with water (4 x 1 ml), and dried to give the 5-(hydroxybenzyl) product (1f) (12 mg, 60%) (yield of unlabelled analog, 80%).

To a stirred suspension of ^{13}C-labelled methyl N-[5(6)-phenylthio-1H-benzimidazol-2-yl]carbamate (fenbendazole) (1d) (30 mg, 0.10 mmol) in 100% acetic acid (1.5 ml) at 45°C, 28% w/w hydrogen peroxide (26 mg, 0.20 mmol) was added. After 45 min at the same temperature when the reaction was completed (TLC), acetic acid was removed under reduced pressure at room temperature. The residue was stirred with methanol (1.5 ml), filtered, washed with diethyl ether, and dried to give the 5-phenylsulfinyl

product (1g) (27 mg in two crops, 84%).

Similar oxidation of ^{13}C-labelled methyl N-[5(6)-propylthio-1H-benzimidazol-2-yl]carbamate (albendazole) (1c) (27 mg, 0.10 mmol) yielded the 5-propylsulfinyl product (1h) (18 mg, 62%).

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