JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2002; **45**: 241–248.
DOI: 10.1002/jlcr.554

Research Article

Synthesis of selectively ^{15}N- or ^{13}C-labelled malachite green

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Summary

Two complimentary syntheses of selectively ^{15}N- or ^{13}C-labelled malachite green hydrochloride were developed in order to provide labelled ligands for structural studies of RNA aptamer/ligand complexes. The ^{15}N- and ^{13}C-labelled versions of the dye have been used in NMR studies to probe the changes in the electronic charge distribution and dynamics of the dye upon binding to RNA. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: ^{15}N-; ^{13}C; aptamer; malachite green; NMR spectroscopy

Introduction

Intramolecular dynamics of both ligand and target are of crucial importance for recognition and stability in complexes between nucleic acids and small molecule ligands.[1–4] NMR spectroscopy is particularly suitable to study structural changes and internal motions on the ns–ms time scale in these systems. In comparison to the large number of studies that look at the nucleic acid dynamics, there are no studies of the ligand dynamics to date. This is, in part, due to the ease with which the nucleic

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Contract/grant sponsor: NSF; Contract/grant number: MCB 0110689
Contract/grant sponsor: NIH; Contract/grant number: RR11973

Received 3 October 2001
Revised 21 November 2001
Accepted 21 November 2001

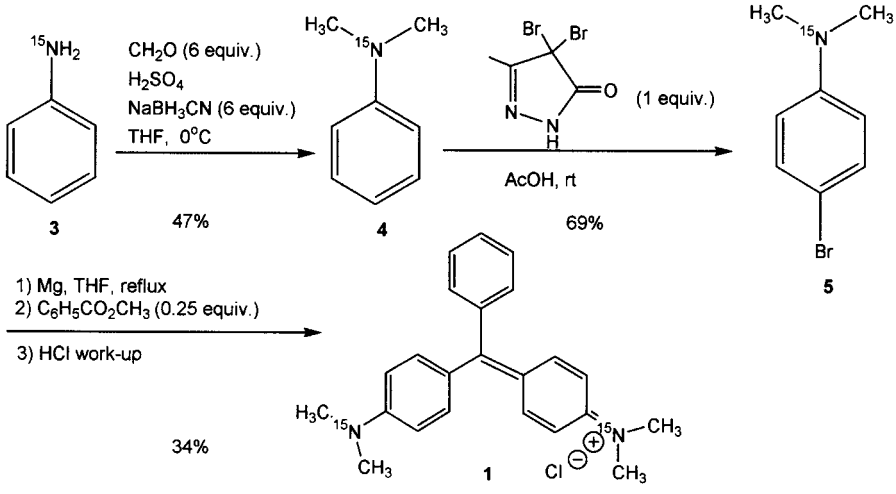
acid can be labelled with NMR active isotopes,[5] whereas the synthesis of labelled ligand is often difficult or extremely costly.

The malachite green binding aptamer is an RNA sequence that binds the organic dye malachite green and related dyes with high affinity. The molecule was identified by *in vitro* selection with the goal to provide a laser activated cleavage site module.[6] Its three-dimensional structure in complex with tetramethylrosamine was determined by X-ray crystallography.[7] However, it was not possible to crystallize the complex with malachite green. NMR studies of the system were initiated in order to determine the structure of the malachite green – RNA complex and to study the dynamics of the dye in the complex.

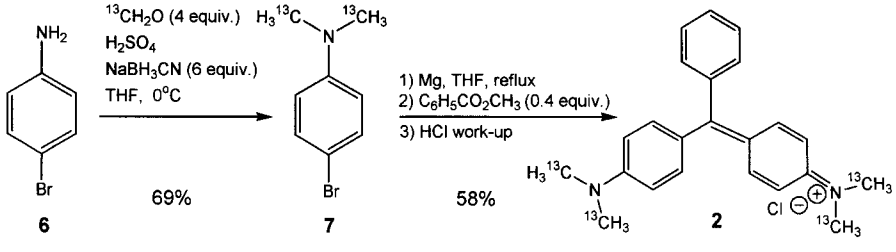
Based on the lack of commercially available labelled malachite green and a limited selection of labelled synthetic precursors, the syntheses of both ^{15}N- and ^{13}C-labelled malachite green were undertaken and are described below. Synthetic precursors had to be labelled in the appropriate place, they needed to be reasonably priced, and had to be materials in which the necessary transformations leading to the dye could be afforded in good yield and high purity. Molecules with labels located in the *N*-methyl groups were chosen because of their central location in the complex and the possibility of using them to characterize conformational exchange processes. The synthetic schemes were initially tested using non-labelled precursors. Emphasis was put on maximizing yields rather than purity because small amounts of impurities are easily identified and do not interfere with the NMR experiments. A preliminary NMR investigation using the synthetic malachite green free and in complex with the RNA aptamer is described below.

Discussion

The synthesis of both the nitrogen and carbon labelled dyes (Schemes 1 and 2) were carried out using ^{15}N-labelled aniline or ^{13}C-labelled formalin purchased from Isotec Inc. (Miamisburg, Ohio). All non-labelled reagents were purchased from Sigma-Aldrich (Milwaukee, Wisconsin). It was possible to shorten the synthesis scheme of the ^{13}C-labelled dye by one step compared to the ^{15}N-labelled dye because unlabelled 4-Bromo-aniline was commercially available at low cost. In addition to the two labelled versions presented here, there are a number of labelled-reagent variations that can be purchased from Isotec Inc. to produce additional labelled versions of malachite green. For example, a



Scheme 1. Synthesis of ^{15}N-labelled malachite green



Scheme 2. Synthesis of ^{13}C-labelled malachite green

fully ^{13}C-labelled version could be synthesized by making the necessary transformations using fully ^{13}C-labelled aniline, formalin and benzoic acid. Additional variations could incorporate labels selectively into rings A and B, or C (Figure 1).

The use of specifically ^{15}N- or ^{13}C-labelled malachite green in multidimensional NMR spectroscopy provides the possibility to probe the electronic structure and internal dynamics of the dye by looking at the ^{15}N- and ^{13}C-chemical shift changes and line widths in the free and bound states. Preliminary NMR studies were performed on the free dye and the RNA/ligand complex (Figure 2) using ^{15}N- and ^{13}C-labelled malachite green. The ^{13}C-labelled dye allows the unambiguous assignment of the methyl groups in the free and the RNA bound dye. The observed changes of the ^{13}C-chemical shifts for the methyl groups in the

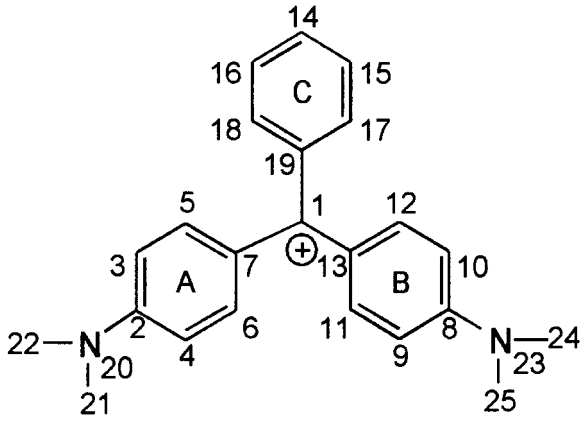


Figure 1. Malachite green with carbon and nitrogen atoms numbered as well as rings labelled to allow discussion of specific labelling

free and bound dye are consistent with a stabilization and thus greater contribution of the malachite green resonance structure that carries the charge on one of the nitrogen atoms. This observation is confirmed by an analysis of the proton line widths for the methyl groups on rings A and B, which indicate a faster rate of rotation for ring B.[8] More detailed NMR studies and simulations of the electron density are currently in progress.

Experimental

Preparation of ^{15}N-labelled N,N-dimethylaniline (4) (Scheme 1): [9,10] A solution containing **3** (0.233 g, 2.5 mmol), $NaBH_3CN$ (0.943 g, 15.0 mmol) and THF (15 ml) was slowly added (over 30 min) to a solution of formalin (0.450 g of a 37% solution, 15.0 mmol) and H_2SO_4 (2.02 ml, 3 M) on ice. The reaction was allowed to continue for 20 h before being basified with 5 M NaOH and subsequently extracted with Et_2O (3×20 ml). The extract was dried over K_2CO_3, filtered and solvent removed by vacuum. The solid/oil was purified by silica gel chromatography (MeOH/EtOAC/Hexane 1:1.5:7.5) separating **4** from monomethylated aniline and **3**, providing a mass of 0.142 g, a 47% yield. The product was used without further purification.

Preparation of ^{15}N-labelled 4-Bromo-N,N-dimethylaniline (5):[11] Selective bromination of the para position in *N*,*N*-dimethylaniline was achieved by placing **4** (0.142 g, 1.17 mmol) in glacial AcOH (2.5 ml) at

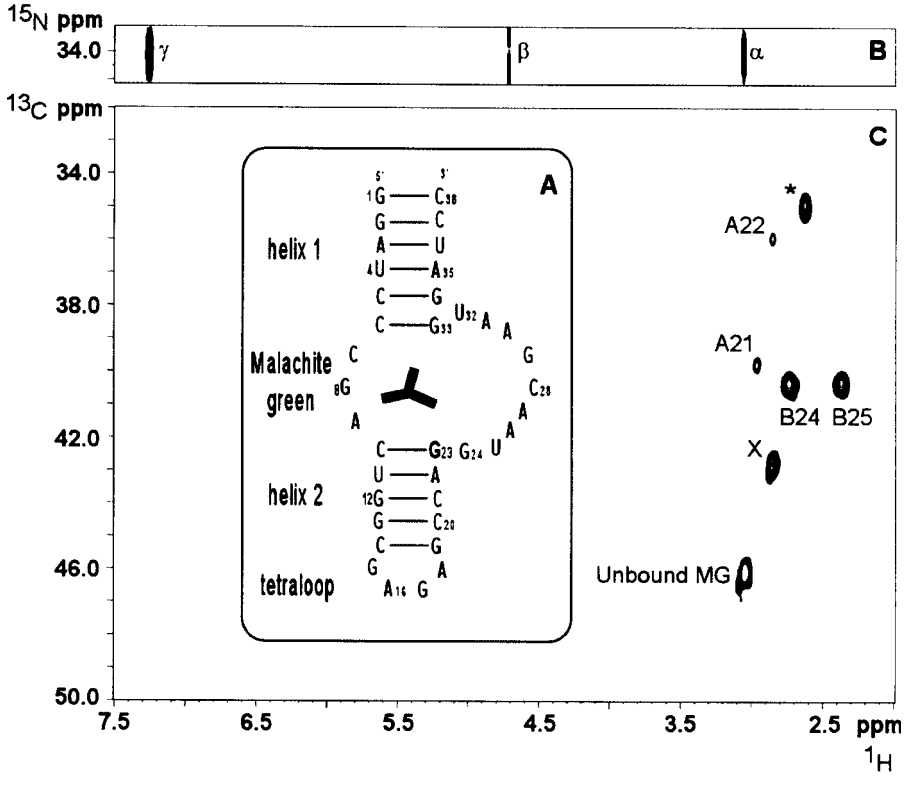


Figure 2. NMR studies of an RNA aptamer with specifically labelled malachite green. (A) Secondary structure of the malachite green binding aptamer. (B) 600 MHz 2D ^{1}H–^{15}N- long-range HSQC correlation spectrum of 1.2 mM ^{15}N-labelled MG dye in D_2O, 10 mM potassium phosphate buffer, 10 mM KCl, pH 5.8 at 25°C. The crosspeak marked α denotes the signal originating from the two-bond coupling between the nitrogens and their respective methyl hydrogens, while γ marks the signal originating from the three-bond coupling between the nitrogens and hydrogens 3, 4 and 9, 10 (Figure 1). β marks the residual HDO resonance. (C) 600 MHz 2D ^{1}H–^{13}C HMQC correlation spectrum in D_2O of ^{13}C-labelled MG dye, 0.8 mM RNA, ratio of RNA : dye 1 : 1.2, 10 mM potassium phosphate buffer, 10 mM KCl, pH 5.8 at 25°C. Signals associated with the four methyl groups are labelled (A21, A22, B24, B25). X and * denote signals originating from residual starting material of the synthesis (4-Br-*N*,*N*-dimethylaniline) and monomethylated dye, a by-product of the synthesis, respectively. The ^{15}N- and ^{13}C-chemical shifts are referenced relative to protons in TMS via the ratios of γ_H to γ_N or γ_C, respectively.[14] The nitrogen chemical shift for the signal in (B) is −353.5 ppm if referenced to nitromethane

25°C, and slowly adding (over 1 h) the brominating agent 4,4-Dibromo-3-methylpyrazol-5-one (0.299 g, 1.17 mmol).[12] After 3 h the solution took on a purple hue with a small amount of white precipitate. At 24 h the color had darkened and amount of precipitate greatly increased. The mixture was filtered and the clear purple supernatant diluted with 30 ml H_2O, neutralized with solid NaOH, extracted with Et_2O (2×15 ml) and CH_2Cl_2 (2×15 ml), dried over K_2CO_3, filtered, and solvent removed. The composition was determined by ^1H NMR. The mixture was purified by silica gel chromatography (MeOH/EtOAC/Hexane 1:1.5:7.5) providing white crystals of mass 0.162 g, a 69% yield.

Preparation of ^{15}N-labelled malachite green hydrochloride (*1*):[13] Fine magnesium ribbon (0.03 g), **5** (0.162 g, 0.810 mmol), and dry THF (3 ml) were refluxed for 1h in a dry flask. The solution was allowed to cool to room temperature before addition of methyl benzoate (0.028 g, 0.025 ml). The solution was refluxed for 10 min then cooled to room temperature and 0.1 ml of 1 M HCl was added, whereupon the solution turned a dark green. The volume of the solution was reduced under vacuum and then purified by column chromatography (Silica gel, 230–400 mesh, 60 Å, *n*-propanol/EtOH/AcOH/H_2O 5/0.33/1/2). A 34% yield was determined by measuring absorbance of malachite green in H_2O at 425 nm using a molar extinction coefficient of 30413 cm^{-1} M^{-1}.[13]

Synthesis of ^{13}C-labelled 4-bromo-N,N-dimethylaniline (7) (Scheme 2): A solution containing **6** (0.143 g, 0.832 mmol), $NaBH_3CN$ (0.310 g, 4.99 mmol) and THF (5 ml) was slowly added to a solution containing ^{13}C-labelled formalin (0.495 g of a 20% solution, 3.33 mmol) and H_2SO_4 (0.337 ml, 6 M) stirring over an ice bath. The reaction was allowed to continue for 20 h before being basified with 5 M NaOH and subsequently extracted with Et_2O (3×15 ml). The extract was dried over K_2CO_3, filtered and solvent removed. This solid/oil was then purified by silica gel chromatography (MeOH/EtOAC/Hexane 1:1.5:7.5) giving white crystals with mass 0.115 g, a percent yield of 69% with respect to **6** and of 17% with respect to the labelled precursor (^{13}C-CH_2O). The product was used without further purification.

Synthesis of ^{13}C-labelled Malachite Green (2): Fine magnesium ribbon (0.02 g), **7** (0.115 g, 0.577 mmol), and dry THF (2 ml) were refluxed for 1 h in a dry flask. The solution was cooled to room temperature before addition of methyl benzoate (0.020 g, 0.0179 ml). The solution was refluxed for 10 min then cooled to room temperature and 0.2 ml of 1 M HCl was added, whereupon the solution turned a

dark green. The volume of the solution was reduced under vacuum and then purified by column chromatography (silica gel, 230–400 mesh, 60Å, *n*-propanol/EtOH/AcOH/H_2O 5/0.33/1/2). A 58% yield was determined by measuring absorbance of malachite green in H_2O at 425 nm using a molar extinction coefficient of 30413 cm^{-1} M^{-1}.

Conclusion

The two synthesis schemes described here provide an easy and inexpensive access to specific NMR probes for the study of ligand structure and dynamics in malachite green RNA complexes. For the purpose of NMR spectroscopy a maximum of material was more important than purity in this case. The bound dye can easily be identified by its NOEs to the RNA and the two major contaminants (residual starting material, 4-Br-*N*,*N*-dimethylaniline and monomethylated dye) do not interact with the aptamer. If necessary the dye can be further purified by column chromatography, albeit at a substantial reduction in yield.

The NMR results outlined above demonstrate that the ^{13}C-labelled methyl groups on rings A and B are in unique electronic environments within the aptamer as becomes apparent by their different ^{1}H and ^{13}C-chemical shifts in the bound state (Figure 2). The use of the specifically labelled dye will allow a first detailed analysis of the ligand dynamics and electronic structure in an RNA aptamer – ligand complex.

Acknowledgements

This work was supported by NSF grant MCB0110689 to T.D. The Bruker 600 MHz spectrometer was paid for in part by funds from NIH grant RR11973. The authors thank Ben Shen and Frank Osterloh for shared equipment, and Janet Trang for technical assistance.

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