Site-specific Mineralization of a Polyester Hydrolysis Product in Natural Soil

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Abstract

Poly(4-methylcaprolactone) (P4MCL) has been successfully incorporated in mechanically competitive materials with potential for biodegradability in engineered and natural systems. The mineralization of the hydrolysis product of P4MCL, 6-hydroxy-4-methylhexanoic acid (**4MHA**), was herein investigated by synthesizing tailormade molecules with ^{13}C-labels in the carboxylic acid group (**4MHA-^{13}COOH**) or in the methyl group (**4MHA-^{13}CH$_3$**) and incubating each separately in a soil. Isotope-sensitive cavity ring down spectroscopy of the efflux gas was then used to quantitatively monitor the mineralization of each isotopomer. These experiments clearly demonstrated that **4MHA** was assimilated and utilized by the soil microorganisms and provided insight into position-specific mineralization. The $^{13}CO_2$ evolution rate profiles and overall extents of mineralization to $^{13}CO_2$ (~85% for carboxyl and ~46% for methyl labeled carbons) are consistent with the methyl carbon being preferentially converted into biomass rather than respired, whereas the carboxyl carbon is preferentially used for energy production and thus mineralized more rapidly (presumably by decarboxylation). These findings agree with previous reports regarding variations in mineralization extents of carbon atoms in different oxidation states. Moreover, this work demonstrates the value of systematically probing biodegradation of polymer hydrolysis products by precise design of ^{13}C-labeled molecules.

Introduction

The amount of plastic estimated to have been discarded as waste since the beginning of industrial plastics production is nearly 5 billion metric tons.[1] Due to the proliferation of mismanaged plastic waste in the environment, polymeric materials susceptible to environmentally relevant degradation mechanisms have garnered significant research attention as one of many approaches to solving this grand societal challenge.[2,3,4] Nonetheless, confusion surrounds communication about the properties of these materials, with terms such as "degradable" and "biodegradable" often having ambiguous meaning.[3] Efforts have been made to standardize these terms, such as the definition of "biodegradable plastic" by ISO/TC61/SC5/WG22 (ISO 472/DAM3, Amendment 3, General Terms and Terms Relating to Degradable Plastics).[5] Central to this definition is that the plastic is completely metabolically utilized by naturally occurring microorganisms (e.g., bacteria and fungi), resulting in the conversion of polymeric carbon to CO_2 (and CH_4 under methanogenic conditions) and to microbial biomass.[6]

Despite the clarity of the definition, polymer biodegradability remains difficult to assess because it is not an intrinsic material property—it is a confluence of both material properties and the (bio)chemical conditions that prevail in a given receiving environment of that material. One of the most pronounced system factors contributing to polymer biodegradation is the local microbial community that is capable of breaking down and of metabolizing the polymeric material.[7,8] In aerobic soil environments, biodegradation typically involves three key steps: first, the colonization of the polymer surface by microorganisms; second, the secretion of extracellular enzymes that can facilitate the breakdown of the polymer chain into low molar mass compounds (provided that abiotic breakdown reactions are slow); and third, the microbial uptake and metabolic utilization of

the low molar mass breakdown products with concomitant formation of CO_2 (referred to as "mineralization") and microbial biomass.[9]

The most conventional techniques to assess biodegradation are respirometry tests, in which the plastic is added to a specific test medium (e.g., soil), followed by incubation during which excess O_2 consumption and/or CO_2 evolution is monitored over time in the plastic-containing medium relative to those in control incubations with plastic-free media.[10] However, these systems are susceptible to error when the added plastic is mineralized at a slow rate, leading to low excess CO_2 formation compared to plastic-free "control" incubations. Additionally, the addition of plastic may result in priming effects (i.e., a plastic-addition induced change in the rate of mineralization of the natural organic matter in the media as compared to the plastic-free control incubations).[10,11] These challenges can be circumvented using ^{13}C-labeled substrates, as the $^{13}CO_2$ evolution can be measured with high sensitivity and ascribed to the added substrate, i.e. clearly delineated from concurrent mineralization of natural organic matter (at normal abundance of ^{13}C).[7,9,10,12] Additionally, position-specific ^{13}C-labeling of carbons in the added analyte can be implemented to obtain insights in differences in the metabolic utilization of carbon atoms in different positions in the analyte.[9,12]

These developments in methods to assess biodegradability provide an important opportunity to employ these new techniques to investigate and understand the fate of new, potentially biodegradable polymers. One pertinent example is poly(4-methylcaprolactone) (P4MCL), an aliphatic polyester that has garnered significant research interest as a candidate for emerging sustainable materials.[13,14,15,16,17,18,19] Its monomeric precursor, 4-methylcaprolactone, can be obtained from biomass or industrial waste products and has potential to be accessed economically on an industrial scale.[20,21,22,23] P4MCL is especially useful in thermoplastic

elastomers (TPEs) and chemically cross-linked elastomers (CCEs) that have been shown to exhibit mechanical properties on par with or better than similar commercial elastomers.[13,14] To probe for biodegradability, the susceptibility of the CCEs to enzymatic hydrolysis was investigated using a cutinase enzyme from a filamentous fungus found in soil (*Fusarium solani*).[14] At all temperatures studied (2–40 °C), the enzymatic hydrolysis proceeded fully to yield the monomeric subunit 6-hydroxy-4-methylhexanoic acid (**4MHA**),[14] which was separately shown to be relatively non-cytotoxic.[24] The end-of-life of P4MCL-derived materials was investigated further in industrial composting conditions, and both the CCEs and TPEs showed high extents of mineralization (>90% and >85% respectively) over 120 days.[24]

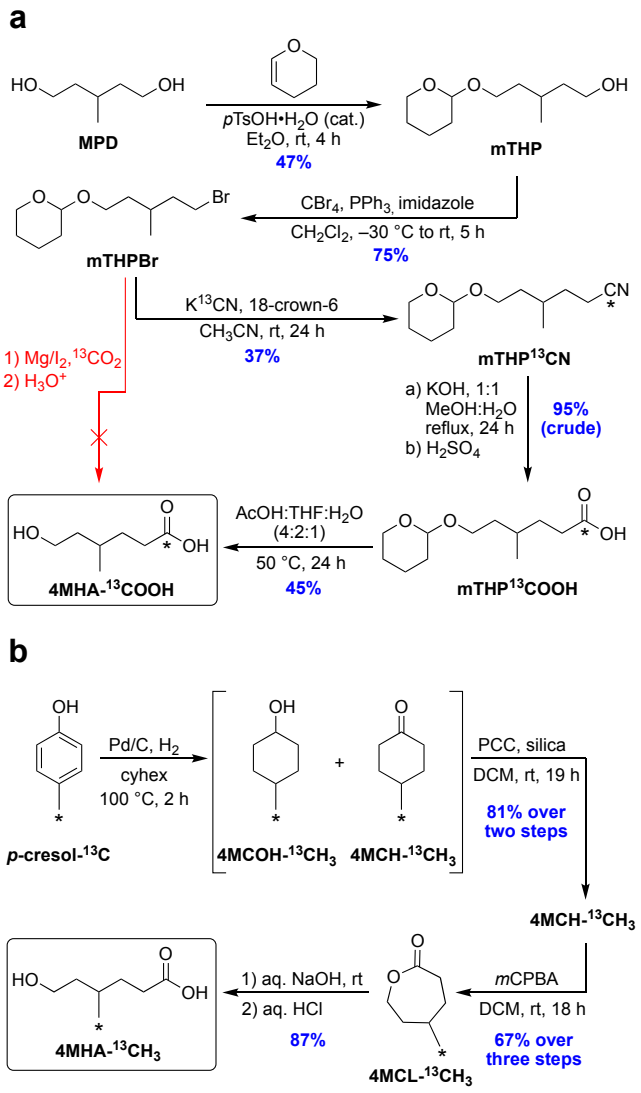
Encouraged by these results, we sought to investigate whether **4MHA** can be readily assimilated and mineralized by microorganisms present in natural soils. We chose soil for these studies as soils are possible receiving environments and because enzymatic hydrolysis of the PMCL elastomer was demonstrated using a soil enzyme (see above). For our test materials, we designed synthetic routes to two different isotopomers of **4MHA**: one with a ^{13}C label at the carboxylic acid group (**4MHA-^{13}COOH**) and the other with a ^{13}C in the methyl group (**4MHA-$^{13}CH_3$**). Separate incubations of each compound in natural soil samples were monitored by ^{13}C-isotope-sensitive cavity ring down spectroscopy (CRDS); the resultant $^{13}CO_2$ evolution rate profiles and cumulative extents of ^{13}C mineralization enabled a comparison of position-specific mineralization of **4MHA**.

Results & Discussion:

Synthesis of 4MHA-^{13}COOH and 4MHA-^{13}CH$_3$

We pursued two routes to synthesize **4MHA-^{13}COOH** from commercially available 3-methyl-1,5-pentanediol (**MPD**) [**Scheme 1a**, **Figures S1–S12**]. Initially we envisioned a Grignard reaction with $^{13}CO_2$ that required protection of one of the alcohols in **MPD**. Installation of a tetrahydropyranyl protecting group provided mono-protected **MPD** (**mTHP**), which was then brominated using an Appel reaction[25] to yield **mTHPBr**; although this compound could reliably be converted to the Grignard reagent, repeated carboxylation attempts failed. The underlying reasons for these failures remain unclear, but we speculate that higher reaction pressures or use of solid $^{13}CO_2$ would result in successful conversion to the desired product. We instead opted to install the isotopic label by nucleophilic substitution of **mTHPBr** with K^{13}CN, which provided **mTHP^{13}CN**. Rather than attempting CN hydrolysis and O-THP deprotection in one step under acidic conditions, we pursued a more controlled two-step procedure wherein **mTHP^{13}CN** was first hydrolyzed under basic conditions. The resultant crude **mTHP^{13}COOH** was then deprotected to obtain the desired product, **4MHA-^{13}COOH** ($\geq$ 95% purity by quantitative ^{1}H NMR spectroscopy).

Scheme 1. Synthetic routes to access (**a**) **4MHA-^{13}COOH** and (**b**) **4MHA-^{13}CH$_3$**.



To acquire **4MHA-^{13}CH$_3$**, we designed a synthetic pathway starting with the catalytic hydrogenation of isotopically-enriched *p*-cresol (***p*-cresol-^{13}CH$_3$**) [**Scheme 1b**, **Figures S13-S18**]. We first tested hydrogenation conditions that selectively yielded the ketone product,[26] but the conversion values of *p*-cresol were very low (< 10%). Quantitative conversion was achieved using elevated hydrogen pressure (100 psig), yielding a mixture of alcohol and ketone (3:1 **4MCOH-^{13}CH$_3$** to **4MCH-^{13}CH$_3$**). We screened several approaches for conversion of **4MCOH-^{13}CH$_3$** to **4MCH-^{13}CH$_3$**, and we ultimately used a modified pydrinium chlorochromate (PCC) oxidation[27] to obtain **4MCH-^{13}CH$_3$**. The cyclic ketone was then subjected to Baeyer-Villiger oxidation[13] to

yield ^{13}C-labeled 4-methylcaprolactone (**4MCL-^{13}CH$_3$**), which was hydrolyzed to obtain the desired **4MHA-^{13}CH$_3$** (≥ 97% purity by quantitative ^{1}H NMR spectroscopy).

Mineralization of ^{13}C-labeled MHAs

We set out to quantitatively evaluate the mineralization rates of the ^{13}C-labeled compounds in a soil collected from an agricultural field by employing an automated soil incubation system. The soil efflux gas is analyzed using isotope-sensitive CRDS to monitor the evolution of $^{13}CO_2$ and $^{12}CO_2$ over time. From these mineralization rate data, the cumulative amount of ^{13}C that is converted into $^{13}CO_2$ can be calculated and compared to the initial amount of ^{13}C-labeled compound added to the soil (**Figure 1**, see associated calculations in Supporting Information). Due to the propensity for these hydroxy-acids to self-condense over time, we stored the purified compounds in freezers at all times and initiated the incubation studies shortly after purification of the target molecules (**Figures S19** and **S20** and associated discussion).

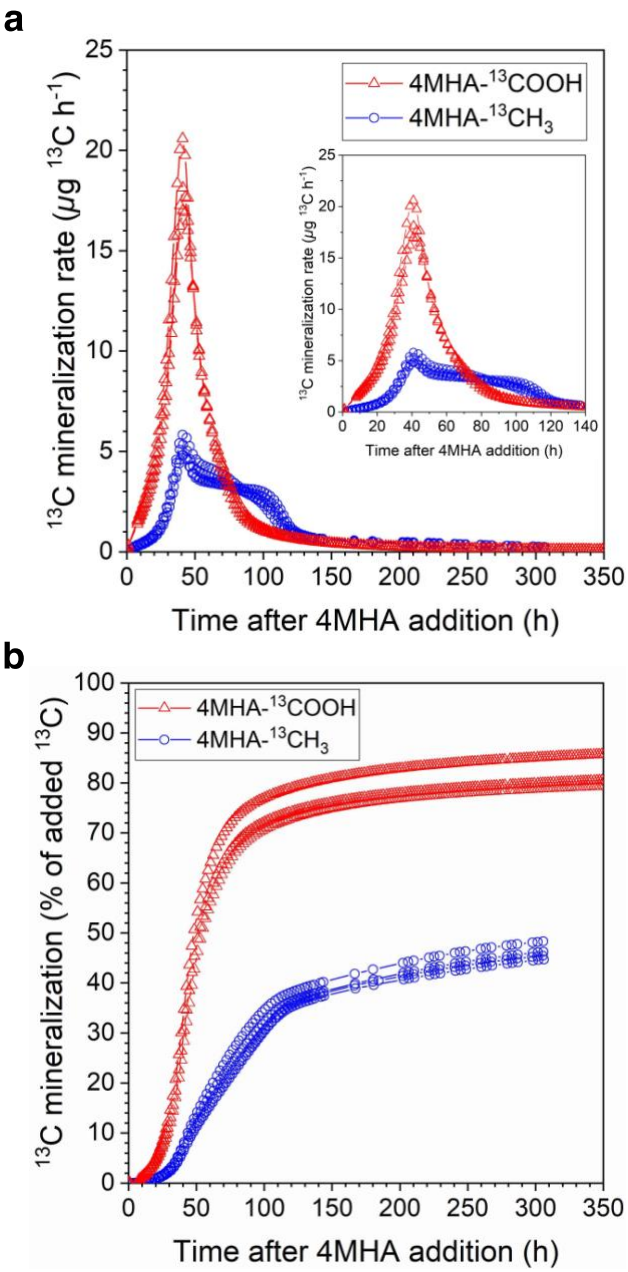


Figure 1. (**a**) Measured mineralization rates and (**b**) calculated cumulative mineralization extents for **4MHA-^{13}COOH** and **4MHA-^{13}CH$_3$**. All replicates performed are shown here (triplicate for **4MHA-^{13}COOH** and quadruplicate for **4MHA-^{13}CH$_3$**), demonstrating the reproducibility of these incubations.

The data shown in **Figure 1** shows different mineralization rate and extent profiles for **4MHA-^{13}COOH** and **4MHA-^{13}CH$_3$**. For **4MHA-^{13}COOH**, a pronounced increase in the rate of $^{13}CO_2$ evolution was observed within hours after addition to the soil, ultimately reaching a

maximum after around 41 h of incubation. After this maximum, a precipitous decrease in the mineralization rate occurred over a similar time frame, ultimately reaching and maintaining very low but measurable rates over the remaining duration of the incubation. This initial burst of $^{13}CO_2$ evolution (0–82 h) was found to account for approximately 70% (range = 67–74%) of the added ^{13}C. The extent of ^{13}C mineralization thereafter continued to increase slowly, reaching a final value of approximately 85% (range = 82–89%) measured at ~38 d (911 h – data shown in **Figure S21**).

For **4MHA-$^{13}CH_3$**, we observed a similar initial increase in mineralization rates with a maximum at approximately 41 h of incubation; however, there were clear differences in the mineralization behavior. The maximum rate was around 4 times lower than for **4MHA-^{13}COOH**, and after achieving the maximum, the rate for **4MHA-$^{13}CH_3$** drops over the next 5 h, thereafter maintaining significant mineralization rates. This second kinetic regime persisted over the next ~60 h, during which the mineralization rates surpass those of **4MHA-^{13}COOH** by up to a factor of 2.5 (**Figure S22**) before dropping to low but measurable values; the final measured rates were still a factor of 2 higher than those of **4MHA-^{13}COOH** (**Figure S22**). The cumulative mineralization extent measured at the end of the incubations (~13 d, 306 h) was approximately 46% (range = 45–48%).

The concurrent rapid initial increases in $^{13}CO_2$ observed for both isotopomers clearly indicate that **4MHA** is rapidly assimilated and utilized by the microbes present in the tested soil. After assimilation, there are two metabolic 'end points' for the substrate carbon: energy production (catabolism) and biomass synthesis (anabolism). The likelihood for specific carbon atoms to be utilized in catabolic processes over anabolic processes has been shown to be largely influenced by their relative oxidation state: highly oxidized carbons typically undergo more extensive mineralization, whereas more reduced carbons are preferentially converted into biomass.[9,12,28,29,30]

All else being equal, a preference for the catabolic utilization over anabolic utilization will manifest as faster mineralization of substrate carbon. As such, the observed variance in the $^{13}CO_2$-respiration profiles presented here provide insight into the position-specific metabolic utilization of **4MHA**—in particular, the relative metabolic fates of the most reduced carbon (i.e., the methyl group) and the most oxidized carbon (i.e., the carboxylic acid group).

In the case of **4MHA-^{13}COOH**, the initial burst of $^{13}CO_2$ suggests that microorganisms rapidly utilize the carboxyl carbon in catabolic processes. The ^{13}C that was not respired during the initial 82 h metabolic window (~30%) was likely converted into microbial biomass. The non-zero rates measured in the later phase (> 82 h) of the incubation are therefore expected to correspond to the relatively slower turnover of substrate-derived ^{13}C-containing microbial biomolecules to $^{13}CO_2$, as compared to initial mineralization of **4MHA-^{13}COOH**. The results for **4MHA-^{13}CH$_3$** incubations provide strong evidence that the methyl substituent of **4MHA** also undergoes extensive microbial mineralization through catabolic processes, despite being the most reduced carbon in the molecule and adjacent to the branch point. However, the emergence of a second kinetic regime after the initial generation of $^{13}CO_2$ and the lower overall extent of ^{13}C-mineralization observed (~46 %) suggest that the metabolic fate of the methyl carbon involves more significant anabolic utilization than the carboxyl carbon. The ^{13}C-mineralization trends from these isotopomers of **4MHA** are consistent with those reported for the mineralization of ^{13}C-labeled succinic acid variants.[12] There are several potential metabolic explanations that can be used to rationalize the more extended mineralization rate profile of **4MHA-^{13}CH$_3$** compared to that of **4MHA-^{13}COOH** (see discussion associated with **Scheme S1** and **Figure S23**). While these results are consistent with the entire **4MHA** molecule being metabolically utilized, our work calls for future studies to characterize the non-mineralized ^{13}C in the soils. Besides quantifying the total

non-mineralized ^{13}C at the end of the incubations to close the ^{13}C mass balance, the ^{13}C-labelling opens the possibility to demonstrate and quantify the extent to which **4MHA** was incorporated into soil microbial biomass. Additionally, future studies should assess responses in the soil microbiome to **4MHA** additions, using additions of readily mineralizable reference molecules known to be non-toxic (e.g., glucose) as controls. These experiments would provide highly relevant ecotoxicological information that are part of typical biodegradation guidelines.[31,32]

Previous work on polyesters has provided strong indication that the rate limiting step of their biodegradation is enzyme-mediated hydrolysis (while the subsequent metabolic utilization of the enzymatic breakdown products was fast). As a consequence, variable mineralization rates observed for position-specific ^{13}C-labeled monomers were 'masked' by slow enzymatic hydrolysis of the corresponding polymers, resulting in absent (or much weaker) position-specificity in mineralization of the monomeric units in the polymeric structure.[9,12] It is therefore likely that in similar soil incubation experiments with isotopically-labeled P4MCL, the relatively slow introduction of assimilable carbon (i.e., **4MHA** monomers and P4MCL oligomers) to the microbial community would result in less pronounced (or even absent) position-specific mineralization rates for P4MCL than were demonstrated here for **4MHA**. Nonetheless, similar studies on the biodegradation of ^{13}C-labeled P4MCL would result in useful complementary information for the more relevant polymeric substrates.

Conclusions

We have prepared two isotopomers of **4MHA** and investigated their mineralization in natural soil samples. Both compounds—**4MHA-^{13}COOH**, labeled at the carboxylic acid carbon, and **4MHA-^{13}CH$_3$**, labeled at the methyl carbon—were synthesized in multiple steps from the

respective precursors: 3-methyl-1,5-pentanediol and isotopically-enriched *p*-cresol. In both cases, soil incubations monitored using ^{13}C-isotope-sensitive CRDS exhibited significant evolution of **4MHA-**derived $^{13}CO_2$ within 5 days, demonstrating that **4MHA** was rapidly assimilated and extensively mineralized by soil microorganisms. Continued, slower mineralization rates were observed throughout the remaining incubation times, indicating mineralization of previously assimilated **4MHA-$^{13}CH_3$** that had been converted into biomass. Furthermore, the incubation studies revealed position-specific mineralization behavior for the two carbons; the carboxylic acid carbon was converted into $^{13}CO_2$ more rapidly and to a higher extent than the methyl carbon. The overall extents of mineralization to $^{13}CO_2$ (~85% for carboxyl and ~46% for methyl) as well as the $^{13}CO_2$ evolution rate profiles over time suggest that the methyl carbon is preferentially used to form biomass rather than respired, whereas the opposite is true for the carboxyl carbon. The finding, however, that also the methyl carbon—the most highly reduced carbon in **4MHA** —is extensively mineralized over these soil incubations suggests that the entire **4MHA** monomeric unit underwent metabolic processing by natural microorganisms. These results strongly support the biodegradability of P4MCL-based materials in soils, given that both their enzymatic hydrolysis by soil esterases as well as microbial utilization of the major enzymatic hydrolysis product by soil microorganisms has now been demonstrated. Viewed in context with related investigations of enzymatic hydrolyzability, composting, and cell toxicity,[14,24] this study demonstrates the value in approaching end-of-life assessment of emerging competitive materials using complementary techniques.

Supporting Information

Information and data regarding chemicals, characterization, syntheses, and mineralization including NMR, FTIR, HRMS, and MALDI-TOF. This material is available free of charge at http://pubs.acs.org.

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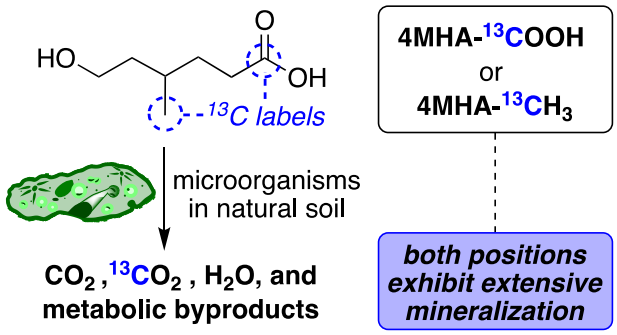

Synopsis

Use of two isotopomers of a polymer hydrolysis product enabled insightful comparisons of position-specific microbial utilization of carbon in biodegradation.

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