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Research Article

An improved synthesis of UDP-3-*O*-acyl-*N*-[^{3}H-acetyl]glucosamine: a probe for inhibitors of LpxC in gram-negative bacteria

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Summary

UDP-3-*O*-(*R*-3-hydroxydecanoyl)-*N*-[^{3}H-acetyl]glucosamine, was prepared using a modified procedure for the preparation of carboxamides reported by Kunishima *et al.* using [^{3}H]CH_3CO_2Na, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, *N*-methylmorpholine and UDP-3-*O*-(*R*-3-hydroxydecanoyl)glucosamine in 26% overall yield. Full details of the synthesis and the analysis of the product are presented. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: [^{3}H]acetylation; inhibitor; DMT-MM; tritium; LpxC

Introduction

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria, and serves as a permeability barrier preventing the penetration of foreign substances through the cell wall. Lipid A is a key component of LPS and serves as the hydrophobic anchor, securing LPS to the outer membrane. The first committed step in the Lipid A biosynthetic pathway is catalyzed by UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase (LpxC). LpxC is an essential enzyme with no known mammalian homolog and is thus an ideal target for antibacterial drug discovery. In order to identify inhibitors of LpxC activity, a radiometric assay described by Kline *et al.*[1] was utilized. A key component of this assay, tritiated LpxC substrate, is not commercially available and had to be synthesized. The published radiosynthesis of UDP-3-*O*-acyl-*N*-[^{3}H-acetyl]glucosamine ([^{3}H-Ac]GlcNac)[1,2] involves a 3 or 4 step procedure to introduce the [^{3}H]acetamide label as shown in Scheme 1. Although the literature synthesis could be used to prepare the product,

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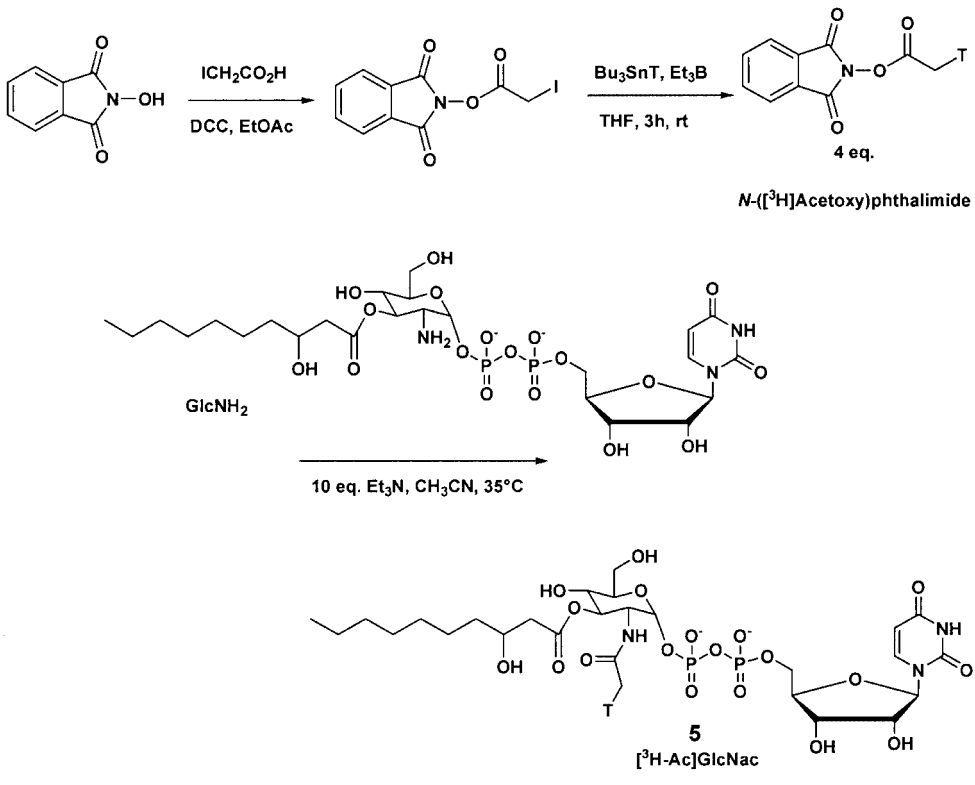


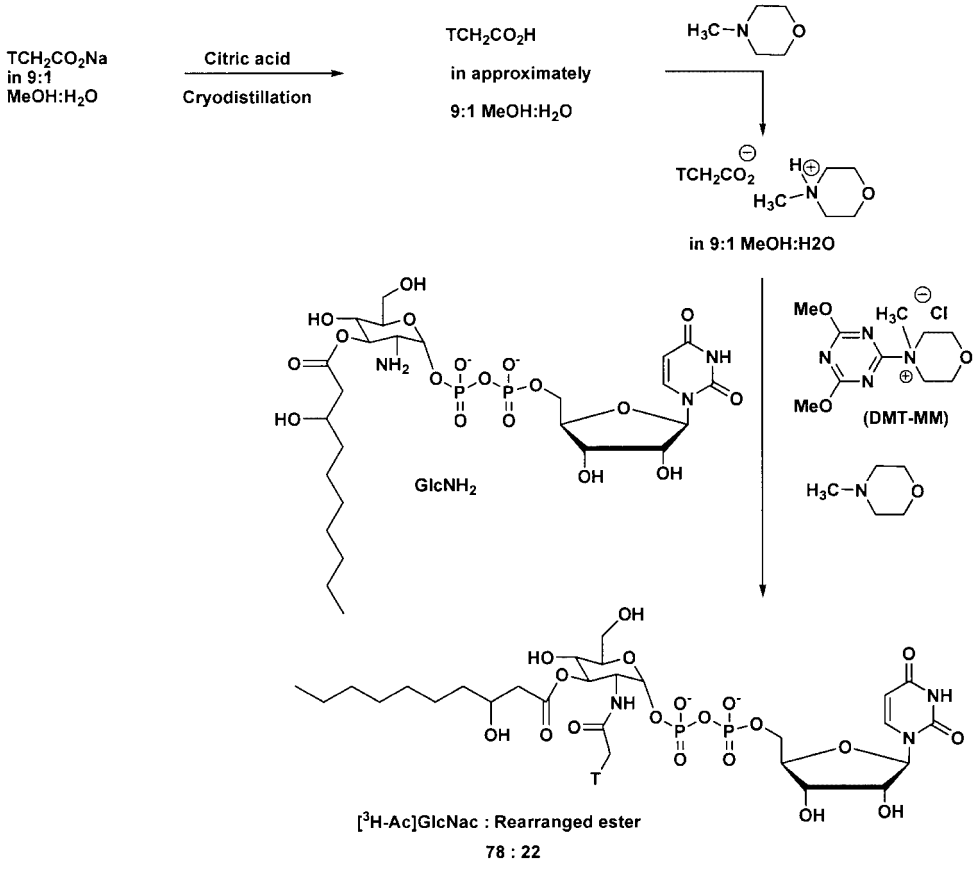
Scheme 1.

repeating it did not seem attractive. First, 4 equivalents of *N*-([^{3}H-Ac]acetoxy)phthalimide are required to introduce the [^{3}H]acetamide group. The remaining 3 equivalents of *N*-([^{3}H-Ac]acetoxy)phthalimide would not be incorporated into the product, but instead would become radioactive waste. Second, *N*-([^{3}H-Ac]acetoxy)phthalimide is prepared in a two-step procedure by the addition of iodoacetic acid to *N*-hydroxyphthalimide followed by tritiodehalogenation with Bu_3SnT.[2] This would require carrying a labeled product through at least one additional step in the synthesis. Third, Bu_3SnT would either have to be purchased or prepared by the tritiation of Bu_3SnLi with T_2O or by the tritiation of Bu_3SnCl with LiT, $LiAlT_4$ or $NaBT_4$.[3] None of these methods seemed attractive, especially considering the large quantities of radioactive waste that would be generated, the additional time required to prepare and analyze Bu_3SnT and the additional safety issues associated with Bu_3SnT. Instead of repeating the published procedures, an improved radiosynthesis of [^{3}H-Ac]GlcNac with commercially available [^{3}H]CH_3CO_2Na, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and *N*-methylmorpholine (NMM) with UDP-3-*O*-(*R*-3-hydroxydecanoyl)gluco-

samine ($GlcNH_2$) following a modified procedure of Kunishima *et al.*[4,5] was developed.

Results and discussion

Kunishima *et al.* reported the use of DMT-MM as an effective agent for the preparation of amides and esters from amines and alcohols with acids or salts of acids in a single step.[4,5] Utilization of this chemistry seemed like an attractive approach for the preparation of [^{3}H-Ac]GlcNac from $GlcNH_2$ and [^{3}H]CH_3CO_2Na as shown in Scheme 2. During the course of developing the reaction conditions for the labeled synthesis, two challenges appeared. First, previous supplies of [^{3}H]CH_3CO_2Na from the supplier contained varying amounts of excess NaOH and it was feared that the batch purchased for the radiosynthesis of [^{3}H-Ac]GlcNac would also contain excess NaOH. Practice reactions with small amounts of NaOH added to CH_3CO_2Na, DMT-MM, NMM and $GlcNH_2$ resulted in decomposition of the $GlcNH_2$ with no



Scheme 2.

formation of product. Therefore, excess NaOH could not be tolerated in the reaction. Second, after 4 days at room temperature, practice reactions would slow down and not go to completion leaving unreacted $GlcNH_2$. Since there was a limited supply of $GlcNH_2$, it was important to convert all of it to the desired labeled product.

The NaOH challenge was overcome by first converting $[^3H]CH_3CO_2Na$ and NaOH to $[^3H]CH_3CO_2H$ and water with excess citric acid and then to the *N*-methylmorpholinium [^{3}H-Ac]OAc salt ($[^3H]CH_3CO_2NMMH$) with NMM in an in-line trap. This solution was added to $GlcNH_2$, DMT-MM and additional NMM. The additional NMM was found to increase the reaction rate. A simplified mechanism for the published reaction involves deprotonation of acetic acid by $GlcNH_2$, followed by attack of the acetate anion on DMT-MM to form an activated intermediate with loss of NMM. The activated acetate is then attacked by $GlcNH_2$ after deprotonation by NMM to form the amide bond of GlcNac. It was reasoned that the reaction became sluggish because either protonated $GlcNH_2$ was not being efficiently deprotonated by NMM or that acetic acid was not being deprotonated effectively. In either case increasing the amount of NMM would seem to increase the rate of reaction. In practice reactions the addition of 1 equivalent of NMM to the reaction resulted in complete reaction of $GlcNH_2$ in approximately one day at room temperature. Using these reaction modifications, 95 mCi of [^{3}H-Ac]GlcNac was prepared in 26% overall yield after purification by reverse phase preparative HPLC. The specific activity was measured by mass spectrometry at 18.1 Ci/mmol (23.3 mCi/mg). The radiochemical purity was measured by reverse phase HPLC at 98.8% and mass spectrometry analysis (ESI, negative ion) showed $m/z = 776$ and 778 for the $M - 1$ and $M + 1$ ions of unlabeled and labeled products. ^{3}H-NMR and ^{1}H-NMR were consistent with that expected for 78% of [^{3}H-Ac]GlcNac and 22% of the rearranged ester resulting from the migration of the acyl group from O3 to O6 that occurred during isolation of the product.[1] Fortunately, the rearranged ester is known to not interfere with the LpxC inhibition assay and there are no known stabilization methods to prevent its migration at this time.

Experimental

Radioactivity was determined using a Packard Model 1900CA Tri-Carb Liquid Scintillation Analyzer. Preparative HPLC was performed on a Waters Delta Prep 4000 HPLC using a Phenomenex LUNA reverse phase column C18, 5 μm, 30 × 250 mm column (gradient conditions: mobile phase A (MP A) = NH_4OAc buffered at pH = 5.6, MP B = Acetonitrile, flowrate = 20.0 ml/min from 0 to 0.5 min, ramping to 40 ml/min at 1.00 min and holding at 40 ml/min for the remainder of the run, linear gradient: 0 min 80%A, 1.0 min 80%A, 20 min 70%A, 22 min 60%A, 24 min 60%A, 27 min 80%A,

UV detection at 254 nm). HPLC/Rad analysis was performed using a HP 1100 HPLC system consisting of a Rheodyne Model 7725i injector, gradient controller, a variable wavelength ultraviolet detector set at 254 nm, a Packard Radiomatic Flo-One Beta Model 500TR radioactivity detector and a Phenomenex LUNA reverse phase column C18, 5 μm, 4.6 × 250 mm gradient: 0 min 80%A, 15 min 32%B, 16 min 40%B, 18 min 40%B, 19 min 80%A. ^{1}H-NMR and ^{3}H-NMR were completed on a Bruker 300 MHz NMR operating at 300 and 320 MHz, respectively. Negative ion electrospray mass spectrometry analyses were completed on a SciEx Model 150 instrument. All labeled materials were identified by HPLC, MS and NMR comparison with the corresponding unlabeled reference materials.

[^{3}H-Ac]GlcNac

To a 5 ml flask with stirbar was added [^{3}H]CH_3CO_2Na (370 mCi, 20 Ci/mmol, 1.5 mg, 18.5 μmol, at 1.0 Ci/ml in 9:1 MeOH:H_2O, American Radiolabeled Chemicals Lot 040130) and citric acid monohydrate (31 mg, 150 μmol). The flask was attached to an in-line trap containing 0.1 M NMM in 9:1 MeOH:Water (185 μl, 18.5 μmol) in the reservoir. The flask and the reservoir were cooled by immersing in liquid N_2. Dissolved gas was removed from the solutions by three freeze, pump, thaw cycles. The contents of the flask were frozen then slowly warmed to room temperature to cryodistill the [^{3}H]CH_3CO_2H into the arm and reservoir of the trap that were immersed in liquid N_2. The final traces of [^{3}H]CH_3CO_2H were distilled by gently heating with a heat gun. The vacuum was released under a positive pressure of N_2(g) and the system was warmed to room temperature. [^{3}H]CH_3CO_2H condensed in the neck of the in-line trap was carefully drained into the reservoir containing the NMM solution and mixed to prepared [^{3}H]CH_3CO_2NMMH in approximately 9:1 MeOH:H_2O. The [^{3}H]CH_3CO_2NMMH solution was transferred to a 2.0 ml reactivial containing $GlcNH_2$ (13.6 mg, 18.5 μmol) and a spinbar. After stirring for 5 min, DMT-MM (5.1 mg, 18.5 μmol) and NMM (2.0 μl, 18.5 μmol) was added and the solution was stirred at room temperature for 23 h. HPLC analysis showed $GlcNH_2$ still remained, so an additional 4.9 mg of DMT-MM was added and the solution was stirred for an additional 4 h at which time no $GlcNH_2$ was present by HPLC analysis. The crude reaction mixture was purified by preparative HPLC. The fractions containing the purified product were immediately neutralized with 0.1 M NH_4OH(aq) to stabilize the [^{3}H-Ac]GlcNac product. The pooled fractions were concentrated by rotary evaporation *in vacuo* at bath temperatures below 30°C until $<$100 ml remained. The volume was adjusted to 100 ml with water to give a stock solution containing 95 mCi of [^{3}H-Ac]GlcNac. HPLC/Rad analysis showed the sample was 98.8% radiochemically pure. Specific activity

was determined to be 18.1 Ci/mmol (23.3 mCi/mg) by mass spectrometry analysis. ^{3}H-NMR (320 MHz, D_2O) analysis showed the presence of two tritium signals at 2.09 and 2.02 ppm in a ratio of 22:78 corresponding to the rearranged ester from migration of the acyl group from O3 to O6 and the desired [^{3}H-Ac]GlcNac. ^{1}H-NMR (300 MHz, D_2O) analysis matched that of the unlabeled standard. Mass spectrometry (negative ion) showed $m/z = 776(80\%)$, 777(37%), 778(100%), 779(30%) and 780(15%). Overall yield for the synthesis was 26%.

Conclusion

A new improved method for the preparation of [^{3}H-Ac]GlcNac was developed using a modification of the procedure of Kunishima *et al.*[4,5] for the preparation of amides.

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