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OleD Loki as a Catalyst for Tertiary Amine and Hydroxamate Glycosylation

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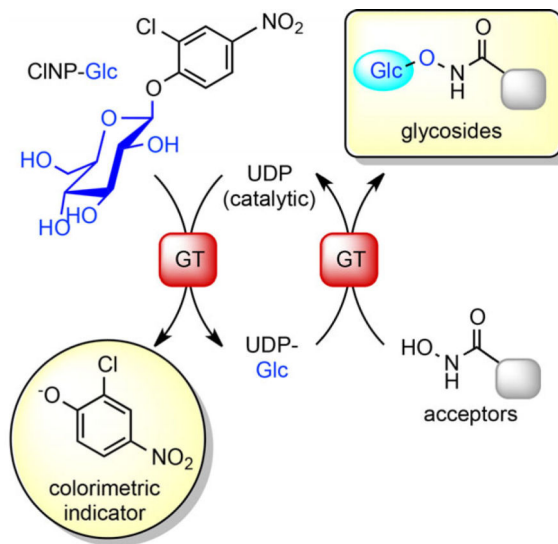
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Abstract

We describe the ability of an engineered glycosyltransferase (OleD Loki) to catalyze the N-glycosylation of tertiary-amine-containing drugs and trichostatin hydroxamate glycosyl ester formation. As such, this study highlights the first bacterial model catalyst for tertiary-amine N-glycosylation and further expands the substrate scope and synthetic potential of engineered OleDs. In addition, this work could open the door to the discovery of similar capabilities among other permissive bacterial glycosyltransferases.

Graphical Abstract



Bacterial model catalyst: The use of a high-throughput colorimetric screen to identify new substrates revealed the engineered macrolide glycosyltransferase OleD Loki as the first reported

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bacterial glycosyltransferase capable of tertiary-amine N-glycosylation. This study also revealed the same enzyme to catalyze trichostatin hydroxamate glycosyl ester formation.

Keywords

antidepressants; drug metabolism; glucuronidation; glycosyltransferases; trichostatin

Glycosyltransferases (GTs), among which sugar nucleotide-dependent GTs (also referred to as Leloir GTs) are the most prevalent, mediate the regio- and stereospecific glycoconjugation of diverse sugars to a broad range of acceptors.^[1] Although Leloir GT-catalyzed formation of O-glycosides is the most common, corresponding microbial GTs involved in the biosynthesis of C-,^[2] S-,^[3] and N-glycosides have also been characterized. Biochemically characterized representative native N-GTs include the indole N-modifying GTs involved in the biosynthesis of indolocarbazoles (AtmG, RebG, NokL, and StaG),^[4] the streptothricin guanidino N-modifying GT StnG^[5] and bacterial protein/ansamitocin asparagine side chain amide-modifying N-GTs (HMWC, NGT, and Asm25).^[6] The gene encoding the putative mannopeptimycin guanidino N-modifying GT has also been reported,^[7] and recent studies of the macrolide-inactivating O-GT OleD,^[8] and corresponding engineered/evolved variants, revealed OleD-catalyzed N-glycosylation of model aromatic/benzyl primary and secondary amines and alkoxyamines.^[9] Although examples of tertiary-amine N-glucuronidation of drugs have been reported as part of phase II metabolism,^[10] to the best of our knowledge, bacterial/fungal comparators are unprecedented. Within this context, herein we describe the discovery that engineered OleDs can catalyze the N-glycosylation of a set of model tertiary-amine-containing drugs and the formation of hydroxamate glycosyl esters. As such, the work put forth further expands the substrate scope and synthetic potential of enhanced OleDs and presents a complement to conventional synthetic strategies to access quaternary amino-N-glycosides.^[11]

Inspired by the general reversibility of GT-catalyzed reactions,^[12] we recently used simple aromatic glycosides as efficient donors in GT-catalyzed sugar nucleotide synthesis and coupled transglycosylation reactions.^[9a, 13] Importantly, the use of 2-chloro-4-nitrophenyl (CINP) glycoside donors in this context also offered a convenient colorimetric screen to enable the directed evolution of enhanced GTs with broad substrate permissivity and the identification of new GT substrates (Figure 1 A).^[9a] Using CINP- β -D-Glc and the OleD variant Loki,^[9a] this colorimetric assay was applied to a panel of 28 representative aliphatic tertiary-amine-containing drugs (Figure S1 the Supporting Information). Assays [1 mM putative acceptor, 2 mM CINP- β -D-Glc, 0.1 mM UDP, 25 mM Tris (pH 8.0), 5 mM MgCl₂, 0.25 μ M OleD Loki, 20 μ L total volume, 30°C, 8 h] were conducted in triplicate in 384-well plates, and progress was monitored through the change in absorbance at 410 nm (A_{410}). Each plate also contained positive (4-methylumbelliferone; 4-MeUmb)^[14] and negative (no acceptor, DMSO) comparator controls. Nine putative primary hits were identified ($A_{410} > 2$ standard deviations above the negative control) in this first-phase screen. Reaction mixtures identified as hits were subsequently subjected to HPLC, desalted,^[15] and analyzed by LC-MS to confirm or refute glycoside formation. This streamlined strategy revealed eight tertiary amines as validated OleD substrates (Figure 1 B), none of which contained

prototypical GT acceptor O-, S-, or N-nucleophiles. To elucidate the nature of the glycosides formed, substrates **1** and **2** were selected for subsequent scale-up and full structure elucidation based on relative turnover in analytical scale reactions (Figure 1C).

Scaled-up reactions for the chemoenzymatic synthesis of the **1** and **2** glucosides were conducted in a total volume of 30 mL [1 mM aglycon, 2 mM C1NP- β -D-Glc, 0.1 mM UDP, 25 mM Tris (pH 8.0), 5 mM MgCl₂, 0.25 μ M OleD Loki, 30°C, 24 h]. Reaction progress was monitored in real time through A₄₁₀, and upon completion, reactants and products were captured by XAD-16 solid-phase extraction, and the resulting glucosides were subsequently purified by HPLC and size-exclusion chromatography. The molecular formulae of the corresponding products were established as C₂₃H₃₀C1N₂O₅S and C₂₅H₃₄C1N₂O₅ by HR-ESIMS; these are consistent with the glucosylation of **1** and **2**, respectively. Interestingly, ¹H and ¹³C NMR spectra of **1a** and **2a** also revealed signatures that are consistent with atypical glucoside formation. Closer analysis revealed 3'-CH₂, 4'-CH₃, and 5'-CH₃ ¹H and ¹³C NMR chemical shifts and clear HMBC correlations that were consistent with quaternary aminoglucosides 3'-N- β -D-glucosylchlorpromazine (**1a**) and 3'-N- β -D-glucosylclomipramine (**2a**; Scheme 1, Tables S1 and S2). Additional COSY and HMBC correlations (Figure S5) were also consistent with this assignment.

The discovery that OleD Loki could catalyze tertiary-amine N-glucosylation prompted a reassessment of additional tertiary-amine-bearing substrates previously identified as OleD Loki substrates—the naturally occurring HDAC inhibitor trichostatin A (**9**);^[16] the c-Raf inhibitor ZM 336372 (**10**)^[17] and the macrolide antibiotic timulcosin (**11**; Scheme 2)^[18]—through colorimetric screening and LC-MS.^[9a] For these selected targets, the strategies employed for reaction scale-up, product isolation, and characterization paralleled those described for **1a** and **2a**. The molecular formulae of the purified glycosides were established by HR-ESIMS to be C₂₃H₃₂N₂O₈, C₂₉H₃₃N₃O₈, and C₅₂H₉₀N₂O₁₈; these are consistent with the glucosylation of **9**, **10**, and **11**, respectively. Unlike for **1a** and **2a**, the ¹H and ¹³C NMR spectra of the isolated glucosides revealed signatures that were consistent with O-glucoside formation. Consistent with this, 1D and 2D NMR data revealed **9a** to be the unique glucopyranosyl hydroxamate trichostatin C and notably highlight the first one-step synthesis of this previously reported rare natural product.^[19] In a similar fashion, the 1D and 2D NMR data revealed **10a** to be 4-O- β -D-glucosyl-ZM336372 and **11a** to be 2'-O- β -D-glucosyltimulcosin, the latter is consistent with the native activity of wtOleD as a macrolide-inactivating glucosyltransferase.^[8b]

To the best of our knowledge, this study highlights the first reported example of a microbial GT that is capable of catalyzing tertiary-amine N-glucosylation. A comparison of the kinetic parameters reveals that the catalytic competencies of tertiary amine **1** ($k_{\text{cat}}/K_{\text{m}}=2.2\times 10^{-4}$ μM^{-1} s⁻¹) and hydroxamate **9** ($k_{\text{cat}}/K_{\text{m}}=1.4\times 10^{-4}$ μM^{-1} s⁻¹) rival that of the parental OleD Loki acceptor 4-MeUmb ($k_{\text{cat}}/K_{\text{m}}=2.2\times 10^{-4}$ μM^{-1} s⁻¹). Although tertiary-amine N-glucuronidation has been reported in the context of phase II metabolism,^[10] fundamental biochemical study of the corresponding glucuronosyltransferases has been hampered by limited access to suitable in vitro models. Likewise, although sugar conjugation is known to influence small-molecule mechanisms, potency, and ADMET,^[1a, e, 20] a lack of practical synthetic or chemoenzymatic access has limited studies to probe the fundamental properties

of quaternary N-glycosides.^[10,11] Our discovery offers a convenient new model for GT-catalyzed tertiary-amine N-glycosylation and a potential complementary synthetic platform for efficient one-step synthesis of quaternary N-glycosides. In a similar fashion, the discovery that OleD Loki is an efficient catalyst for hydroxamate glycosyl ester synthesis further extends the demonstrated synthetic utility of this permissive catalyst. Analysis of the established substrates **1–8** highlights a common alkyl N,N-dimethyl acceptor nucleophile that extends from a hydrophobic aromatic core reminiscent of the longer unsaturated spacer separating the **9** pharmacophore and warhead or the previously reported ability of OleD ASP to catalyze asymmetric 4'-O-glycosylation of one "arm" of mitoxantrone.^[21] The lack of detectable tertiary-amine N-glycosylation with substrates **9–11** may be attributed to substrate orientation in the enzyme-bound complex (as suggested by the determined OleD:macrolide complex, Figure 2) and/or poor nucleophilicity (as anticipated in the context of the *N,N*-dimethyl aniline moiety of **9** and **10**).^[22] Although these examples suggest that certain substrate specificity features might infringe on OleD Loki-catalyzed tertiary-amine reactant scope, the underlying discovery also importantly implicates *N,N*-dimethylamino- and/or hydroxamate-functionalized small molecules as a potential acceptors to evaluate in the context of other permissive GTs.^[23]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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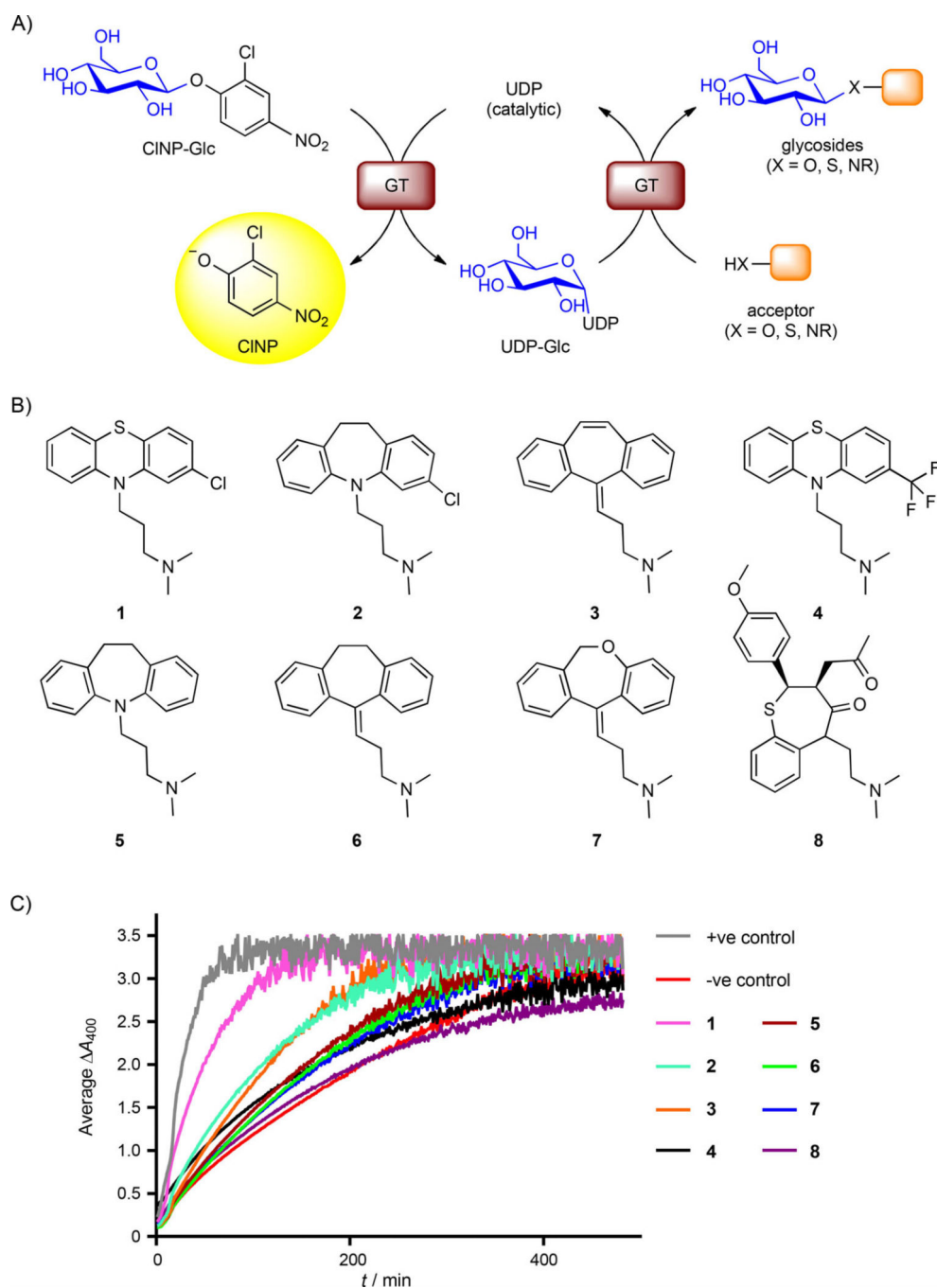


Figure 1. A) Schematic of colorimetric high-throughput screen. B) Tertiary-amine-containing pharmacophores identified as putative OleD Loki substrates: chlorpromazine (1), clomipramine (2), cyclobenzaprine (3), triflupromazine (4), imipramine (5), amitriptyline (6), doxepin (7), and diltiazem (8). C) Reaction progress as monitored by ΔA_{410} in the standard 50 mL assay format [see (A) and main text]. Vehicle without acceptor served as the negative control, and 4-MeUmb served as the positive control. Assays were conducted in triplicate with less than 5% error between replicates.

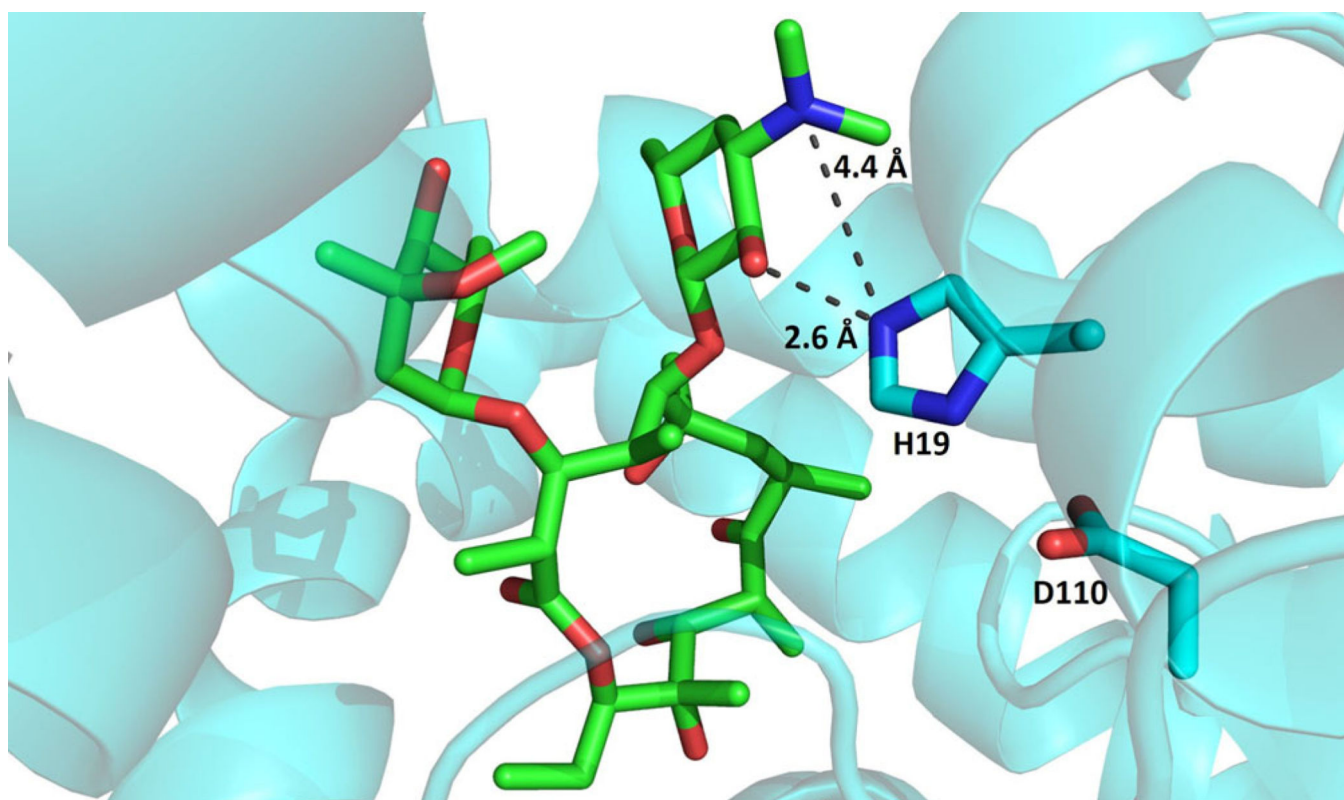
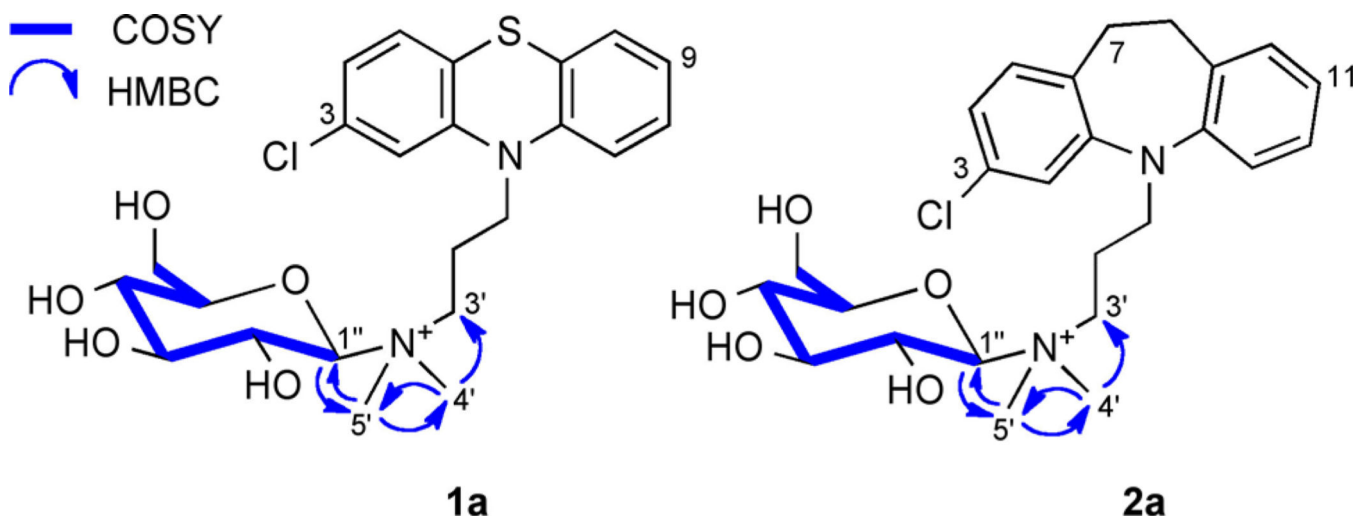
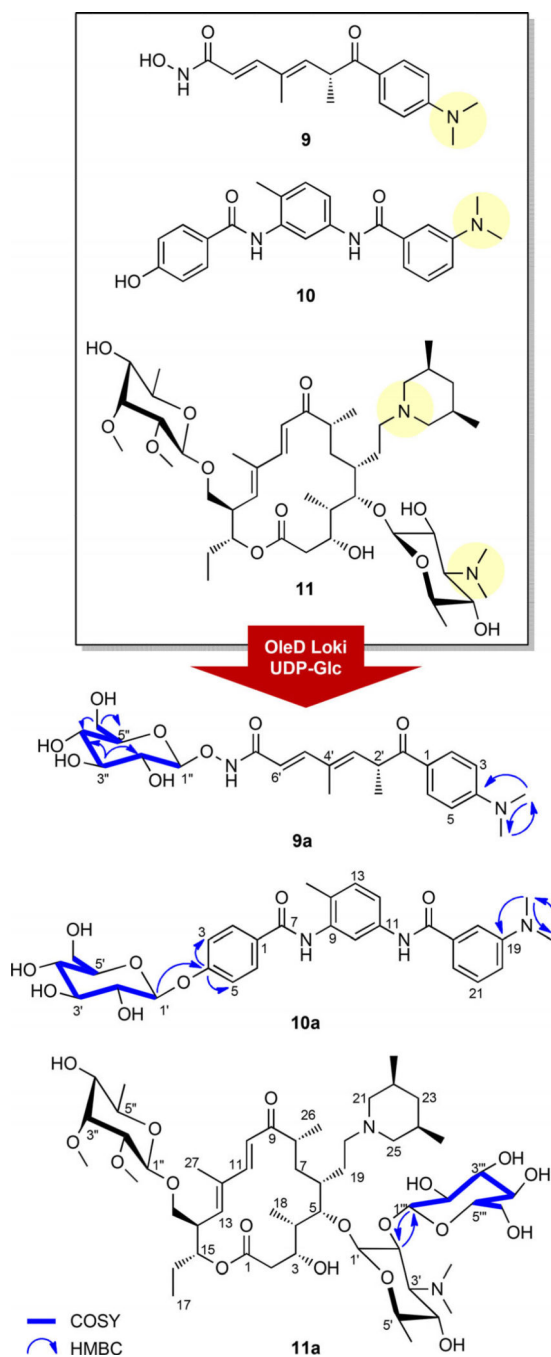


Figure 2. Crystal structure of wtOleD bound to erythromycin (PDB ID: 2IYF). Dotted lines highlight the proximity of the known acceptor nucleophile (desosamine 2'-OH, which corresponds to the mycaminoses 2'-OH in **11**) and the closest tertiary amine (desosamine 3'-N, N-dimethyl, which corresponds to the mycaminoses 3'-N, N-dimethyl in **11**) to the histidine 19 side chain within the active-site H19-D110 acid–base pair.

**Scheme 1.**Key HMBC and COSY correlations for compounds **1a** and **2a**.

**Scheme 2.**

Additional tertiary-amine-containing OleD substrates trichostatin A (**9**), ZM 336372 (**10**), and tilmicosin (**11**) and their products. Tertiary amines within **9–11** are shaded yellow, and key HMBC and COSY correlations are highlighted.