Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 1583–1592

BIOORGANIC & MEDICINAL CHEMISTRY

Combinatorial Enzymatic Assay for the Screening of a New Class of Bacterial Cell Wall Inhibitors

Ahmed El Zoeiby,[a,†] Mélanie Beaumont[b,†] Eric Dubuc,[b] François Sanschagrin,[a] Normand Voyer[b] and Roger C. Levesque[a,*]

[a]*Centre de recherche sur la fonction, structure et ingénierie des protéines, Faculté de médecine, pavillon Charles-Eugène-Marchand, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4*
[b]*Centre de recherche sur la fonction, structure et ingénierie des protéines, Département de chimie, Faculté des sciences et génie, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4*

Received 1 July 2002; accepted 9 September 2002

Abstract—We have developed a screening assay by thin-layer chromatography (TLC) to identify inhibitors for the bacterial essential enzymes MurA, -B, and -C. Libraries of compounds were synthesized using the mix-and-split combinatorial chemistry approach. Screening of the pooled compounds using the developed assay revealed the presence of many pools active in vitro. Pools of interest were tested for antibacterial activity. Individual molecules in the active pools were synthesized and retested with the TLC assay and with bacteria. We focused on the best five compounds for further analysis. They were tested for inhibition on each of the three enzymes separately, and showed no inhibition of MurA or MurB activity but were all inhibitors of MurC enzyme. This approach yielded interesting lead compounds for the development of novel antibacterial agents.

Introduction

Back in the 1940s and 1950s, when the first antibiotics such as penicillin began making their way into clinical use, they were regarded as miracle drugs. However, the widespread use and misuse of antibiotics since their introduction into the medical practice led to an undesired effect which is the selection for antibiotic resistant pathogens. That is because this overuse of antibiotics imposes immense selective pressures for the emergence of such resistant bacteria. This increased resistance, being a serious threat to antimicrobial therapy, necessitates the development of novel antibacterial agents. The machinery for peptidoglycan biosynthesis is an ideal site for antibacterial targets, since it is essential for bacterial survival and it has no mammalian counterpart. Many antibiotics in clinical use interfere with the polymerization steps of peptidoglycan. However, earlier cytoplasmic steps of peptidoglycan biosynthesis, catalyzed by Mur enzymes, are not sufficiently exploited as antibiotic targets. Since the Mur enzymes are highly conserved among different bacterial species,[1,2] inhibitors of any of these enzymes would possess a broad spectrum of action, which presents another argument validating the choice of the Mur pathway as a candidate for antibacterial drug development.

The first committed step in peptidoglycan biosynthesis is the transfer of an enolpyruvate moiety from phosphoenolpyruvate (PEP) to the 3-OH of the glucosyl group of UDP-*N*-acetylglucosamine, a reaction catalyzed by MurA. This is followed by the reduction of the enolpyruvate residue to a lactate residue by the MurB enzyme, and the sequential addition of the amino acids forming the peptide bridge of peptidoglycan by the action of MurC through F enzymes.[3,4] Among all these essential enzymes, only MurA is a target of a known antibiotic, Fosfomycin,[5] which irreversibly inactivates MurA in a time-dependent manner by covalently modifying Cys 115.[6,7] Recently, many attempts have been made to find out novel inhibitors of the Mur pathway. Inhibitors for MurA,[8] MurB,[9] MurC,[10,11] MurD,[12–16] and MurE[17,18] have been described.

With the continuous development of high throughput screening methods, there is a growing need for the synthesis of a large number of molecules. Combinatorial chemistry responds to that growing need. It is a technology allowing the quick generation of huge numbers of structurally related compounds.[19] Combinatorial chemistry approach can be achieved either in solution or in solid-phase. We decided to build our libraries using solid support combinatorial

*Corresponding author. Tel.: +1-418-656-3070; fax: +1-418-656-7176; e-mail: rclevesq@rsvs.ulaval.ca
†Both authors have contributed equally to this work.

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PII: S0968-0896(02)00447-9

chemistry since it presents many advantages: reactions can be accelerated by using excess reactants; the use of such excess favours a complete reaction; washing and purification steps, often troublesome, are much facilitated; and the process can be easily automated, which permits obtaining higher yields and considerable time saving.[20]

In this paper, we describe the development of a novel screening assay for cell wall inhibitors and the chemical synthesis of libraries of amino acid derivatives as potential Mur inhibitors. We used the mix-and-split approach to synthesize our libraries of molecules, then we adopted the parallel synthesis technique to synthesize the unique compounds in the libraries of interest.

Results and Discussion

Purification of *P. aeruginosa* MurA, -B and -C proteins

P. aeruginosa murA, -B and *-C* genes were cloned in the pET 30a expression vector as described elsewhere,[21,22] adding six Histidine tag fusions to their C-termini. Overexpressed proteins were purified in mg quantities to homogeneity via their His tags by a single chromatographic step on an affinity nickel column. Protein identities were confirmed by N-terminal sequencing of the first 15 amino acid residues.

Development of a screening assay for cell wall inhibitors

A novel combinatorial screening assay for MurA, MurB and MurC inhibitors was developed. The ligase reaction catalyzed by MurC (UDP-*N*-acetylmurmate:L-alanine ligase) drives its energy from the hydrolysis of ATP into ADP and inorganic phosphate. In presence of purified MurA, -B and -C enzymes, PEP, NADPH, L-alanine, and ATP, the MurA substrate UDP-*N*-acetylglucosamine is converted into UDP-*N*-acetylmuramyl-L-alanine with a complete hydrolysis of ATP into ADP and inorganic phosphate (Fig. 1). In the presence of an inhibitor of any of the 3 enzymes, ATP consumption in the reaction is reduced or completely annihilated. This inhibition can be detected by using α-^{32}P radioactively labelled ATP in the reaction mixture, then separating ATP from ADP by TLC. The extent of this inhibition is proportional to the decrease in % of ATP hydrolysis, and hence could be quantitated. The radioactive spots corresponding to ATP are compared to those corresponding to ADP and the % of ATP hydrolysis for each tested compound is calculated using the program Image Gauge.

Combinatorial chemistry approach for the synthesis of inhibitors

We have synthesized more than 430 different molecules using the mix-and-split combinatorial chemistry approach (Fig. 2). The central nucleus of these molecules was a D-amino acid, in order to reduce the interference with the metabolic pathways of eukaryotic cells. The intended compounds were highly diversified small molecules. That is why we modified both N and C terminals of the central amino acid. The 432 synthesized molecules were grouped into 6 libraries that could be divided each into 12 sub-libraries. Each of the sub-libraries contains 6 different compounds. In

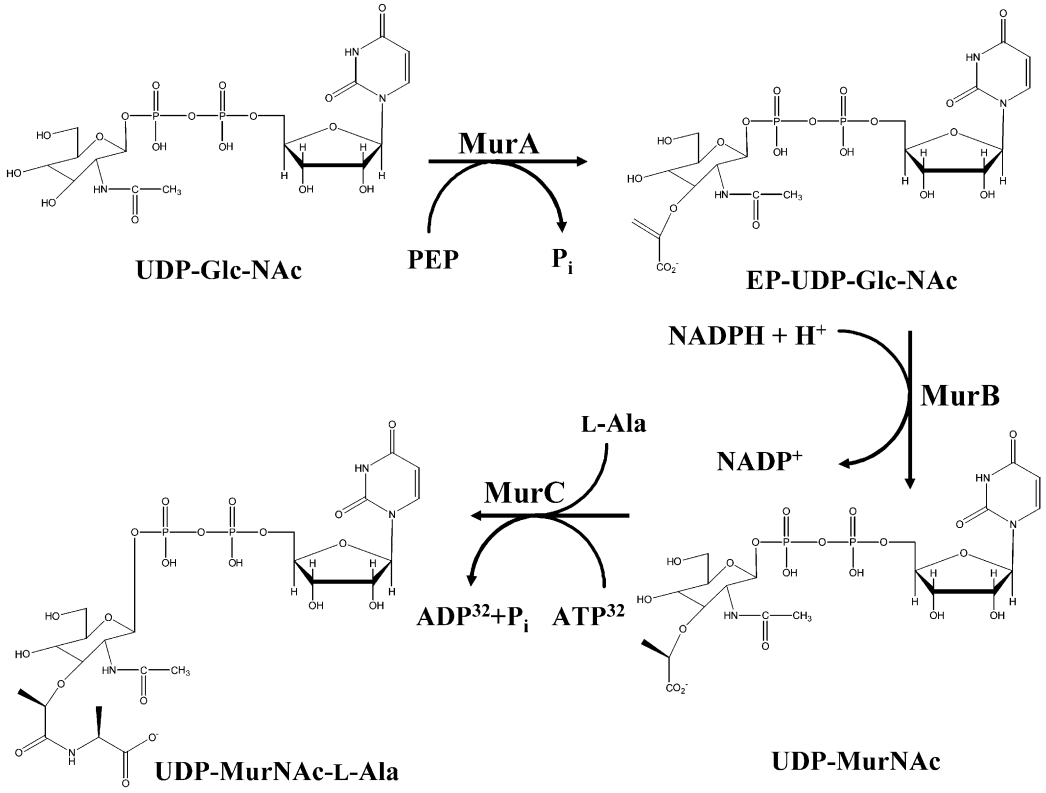

Figure 1. MurA, -B and -C catalyzed reactions used in the ATP [α-^{32}P]-based TLC assay for the screening of cell wall inhibitors.

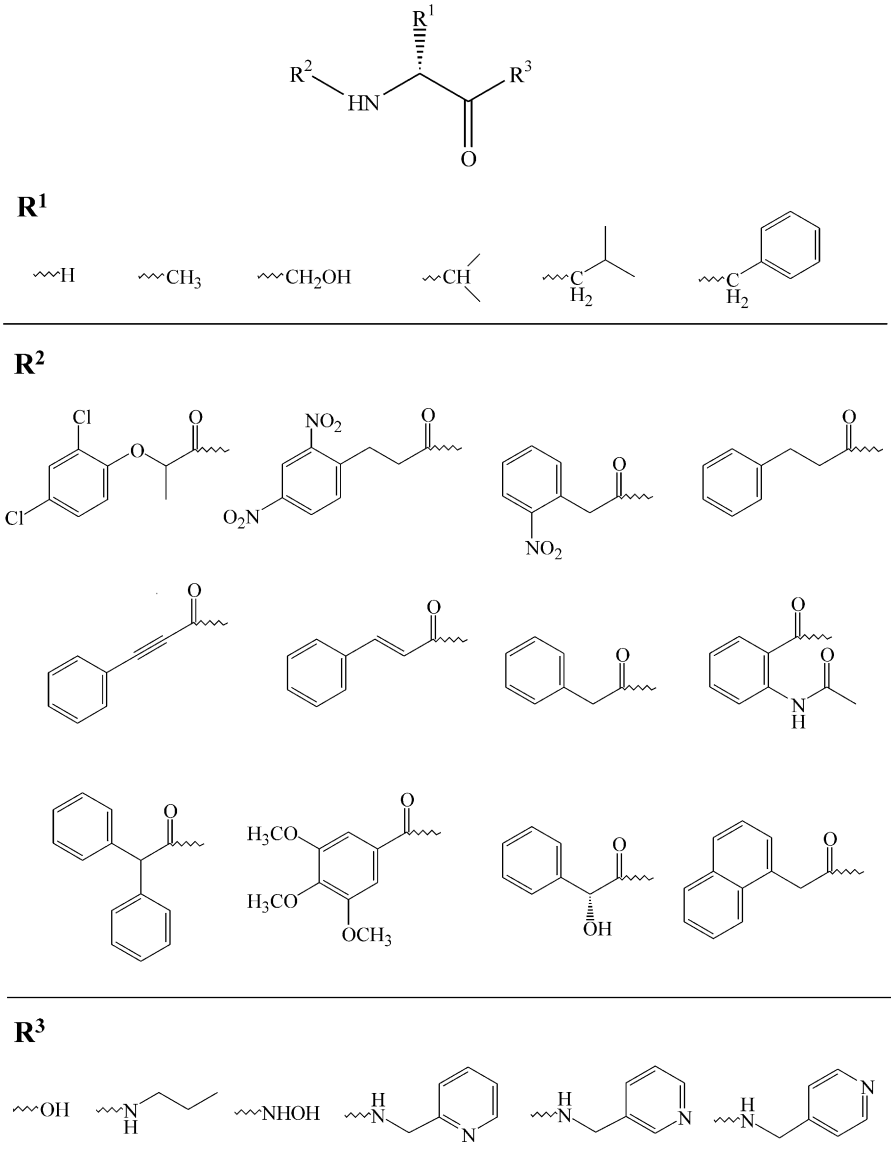

Figure 2. General formula and different side chains of the combinatorial libraries synthesized by the mix-and-split approach and screened for cell wall inhibitors.

order to synthesize these numerous molecules, six different D-amino acids were used (R^1). Then, 12 different chemical groups were used to modify the functionality of the N-terminal (R^2). All of these R^2 groups were aromatic. This choice was based on two reasons. First, among the numerous antibiotics already present in the market, many possess an aromatic pharmacophore. Second, since an HPLC analysis is performed on each library, the presence of an aromatic chromophore facilitates the detection of each product using a UV detector. However, it is to be noted that a multitude of other reactants could be used, such as acid chlorides, isocyanates or chlorosulfonates. Finally, 6 different nucleophiles were used to modify the functionality of the C-terminal. These nucleophiles (R^3) allow the preparation of diversified libraries and a rapid analysis of structure–activity-relationships. Hydroxamates formed by the cleavage with hydroxylamine possess an inhibitory action on other types of enzymes,[23–26] which makes their testing on Mur enzymes more interesting.

Testing of the synthesized compounds in vitro and on bacteria

Screening of the 432 compounds grouped into 72 pools of 6 using the ATP-based assay revealed the presence of 54 pools inhibiting ATP hydrolysis in the coupled enzymatic assay. Molecules of interest were tested for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* by the agar diffusion assay described below. Among the pools active in vitro, 14 showed antibacterial activity. The 84 (14×6) molecules in the active pools were synthesized individually by a parallel solid-phase synthesis, using the same chemical procedure. The individual compounds were then tested with the ATP-based assay (Fig. 3) and with the drug susceptibility testing (data not shown). The most active molecules possess distinct structural features such as (*R*)-(−)-mandelic acid at the R^2 position (modification of N-terminal) and 3- or 4-(aminomethyl)-pyridine at the R^3 position (modification of C-terminal and cleavage).

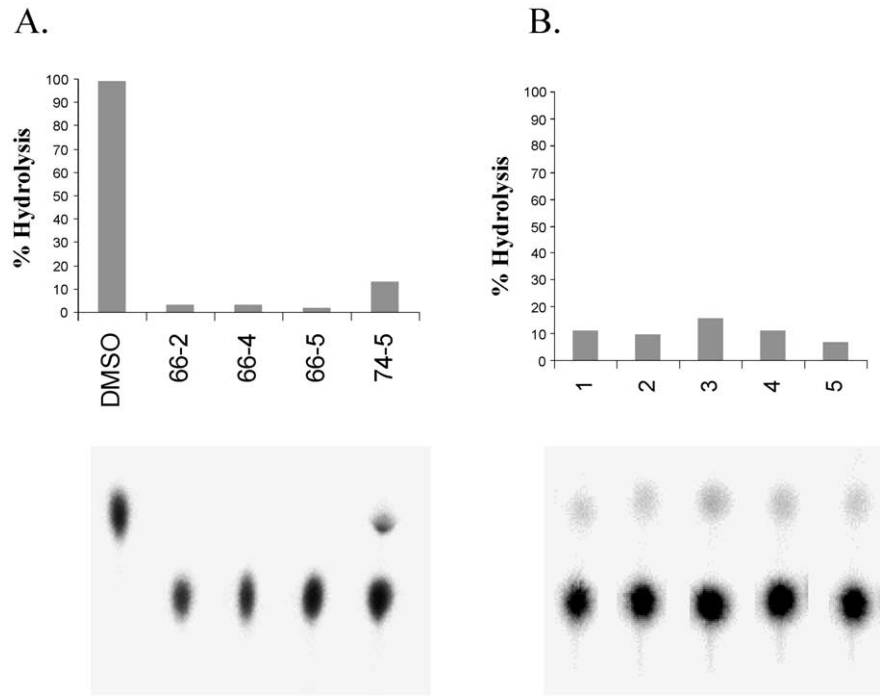

Figure 3. TLC screening and % of ATP [α-^{32}P] hydrolysis obtained with selected individual compounds (B) and their corresponding pooled compounds (A).

The five compounds showing the best inhibitory activity both in vitro and on bacterial cells were selected for further analyses.

Confirmation of the chemical structure of the active compounds

Three of the 5 molecules (Fig. 4) were analyzed by NMR and mass spectrometry in order to validate the exactitude of chemical synthesis. ^{1}H NMR and LC/MS analysis confirmed the expected structure of each compound. In mass spectrometry analysis, the molecular ions $(M+H)^+$ were present in large abundance compared to the $(M+Na)^+$ ions, and all corresponded to the exact calculated mass of the compounds. In the ^{1}H NMR analysis, each proton was detected with a good chemical shift and integration. On the other hand, a few coupling constants were not clear due to a lower resolution of spectrum and signal overlaps, but the obtained NMR data were sufficient to strongly confirm the chemical structures of the molecules.

NMR and MS data

***N*-(*R*)-(−)-Mandelyl-D-leucyl-4-(aminomethyl)-pyridine (2).** HPLC: $R_t = 20.7$ min. MS (API-ES): m/z $356.3 = (M+H)^+$, $378.1 = (M+Na)^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.78 (d, 3H, $J = 5.5$ Hz, CH_2–CH(C**H$_3$**)$_2$), 0.86 (d, 3H, $J = 5.6$ Hz, CH_2–CH(C**H$_3$**)$_2$), 1.49 (m, 1H, CH_2–C**H**(CH$_3$)$_2$), 1.58 (m, 2H, C**H$_2$**-CH(CH$_3$)$_2$), 3.60 (d, 2H, $J = 6.7$ Hz, C**H$_2$** aminomethyl), 4.38 (m, 1H, C**H$_\alpha$**), 4.99 (s, 1H, C**H**-OH), 6.26 (m, 1H, **OH**), 7.27–7.48 (m, 8H, **H** arom), 8.02 (d, 1H, $J = 8.6$ Hz, N**H$_\alpha$**), 8.53 (d, 1H, $J = 5.5$ Hz, **H** aminomethyl), 8.62 (t, 1H, $J = 5.8$ Hz, N**H** aminomethyl).

***N*-Diphenylacetyl-glycyl-3-(aminomethyl)-pyridine (4).** HPLC: $R_t = 22.6$ min. MS (API-ES): m/z $360.1 = (M+H)^+$, $382.1 = (M+Na)^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.80 (d, 2H, $J = 5.6$ Hz, C**H$_2$** gly), 4.32 (d, 2H, $J = 5.7$ Hz, C**H$_2$** aminomethyl), 5.08 (s, 1H, C**H**), 7.23 (m, 1H, N**H**), 7.31 (s, 10H, **H** arom), 7.64 (d, 1H, $J = 7.6$ Hz, **H** aminomethyl), 8.47 (m, 2H, **H** aminomethyl), 8.54 (m, 1H, N**H** aminomethyl).

***N*-(*R*)-(−)-Mandelyl-D-leucyl-3-(aminomethyl)-pyridine (5).** HPLC: $R_t = 20.7$ min. MS (API-ES): m/z $356.1 = (M+H)^+$, $378.1 = (M+Na)^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.78 (d, 3H, CH_2–CH(C**H$_3$**)$_2$), 0.86 (d, 3H, CH_2–CH(C**H$_3$**)$_2$), 1.50 (m, 1H, CH_2–C**H**(CH$_3$)$_2$), 1.58 (m, 2H, C**H$_2$**–CH(CH$_3$)$_2$), 4.34 (m, 1H, C**H$_\alpha$**), 4.36 (m, 2H, C**H$_2$** aminomethyl), 4.98 (s, 1H, C**H–OH**), 6.29 (s, 1H, CH–O**H**), 7.28–7.43 (m, 8H, **H** arom), 7.63 (d, 1H, $J = 6.9$ Hz, N**H$_\alpha$**), 8.47 (m, 1H, **H** aminomethyl), 8.59 (m, 1H, N**H** aminomethyl).

Enzymatic synthesis and purification of pathway intermediates. Substrates of the Mur pathway are not commercially available, except for the MurA substrate, UDP-*N*-acetylglucosamine. In order to test the 5 chosen compounds separately on the MurB and the MurC catalyzed reactions, we needed to synthesize and purify the MurB substrate UDP-*N*-acetylglucosamine-enolpyruvate, and the MurC substrate UDP-*N*-acetylmuramic acid. Purified MurA protein was used to synthesize the MurB substrate, and purified MurA and MurB proteins were used in combination to synthesize the MurC substrate, starting from MurA substrate in both reactions. Control and enzymatic reactions were analyzed by FPLC and collected fractions of the peaks were analyzed by mass spectrometry. In case of

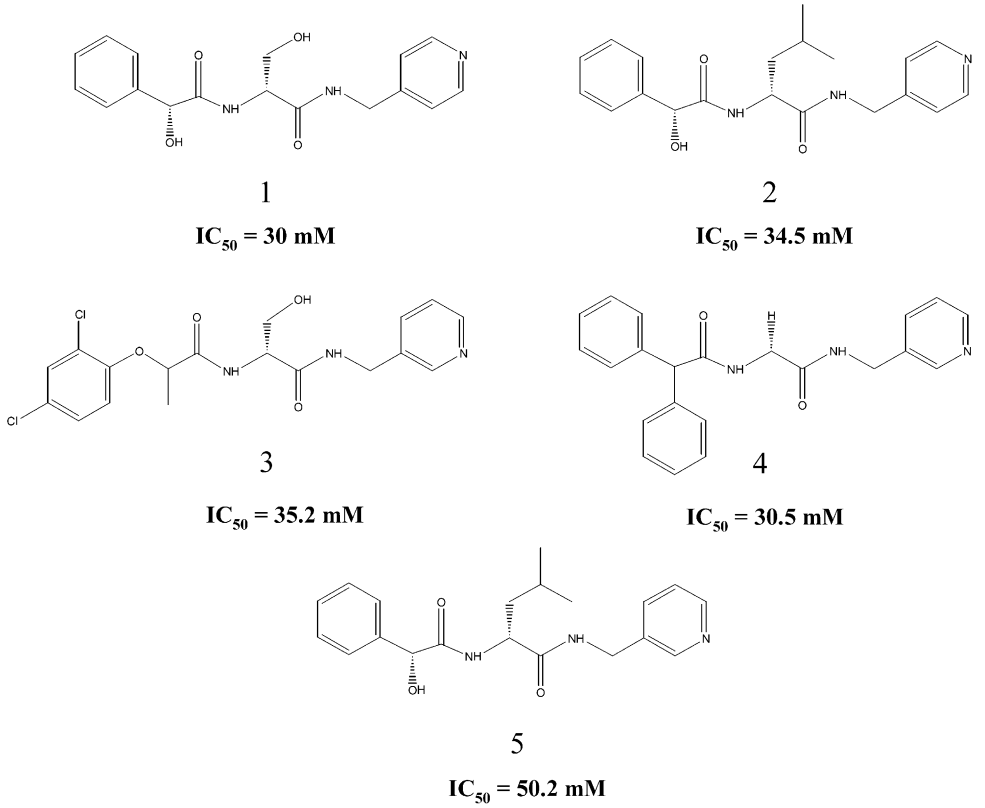

Figure 4. Chemical structure and IC_{50} values of the inhibitors **1**, **2**, **3**, **4** and **5**.

UDP-*N*-acetylglucosamine-enolpyruvate synthesis, the peak eluted at 36 min was the only peak present in the enzymatic reaction and absent in the control, and in case of UDP-*N*-acetylmuramic acid synthesis, it was the peak eluted at 32 min. Both peaks were analyzed by mass spectrometry. The peak eluted at 36 min yielded a major peak with *m*/*z* 274.1 (Fig. 5A) corresponding to the calculated mass of the *N*-acetylglucosamine-enolpyruvate moiety, after the loss of the UDP moiety and the gain of one proton: $C_{11}H_{16}N_1O_7$. The peak eluted at 32 min yielded a major peak with *m*/*z* 276.1 (Fig. 5B) corresponding to the calculated mass of the *N*-acetylmuramic acid moiety, after the loss of the UDP moiety and the gain of one proton: $C_{11}H_{18}N_1O_7$.

Inhibition assays on MurA and MurB

The molecules **1**–**5** were tested for inhibition on the MurA and the MurB catalyzed reactions separately. For the MurA inhibition assay, enzymatic activity was assessed by quantitating the UDP-*N*-acetylglucosamine dependent release of inorganic phosphate from PEP. For the MurB inhibition assay, enzymatic activity was assessed by monitoring the decrease of NADPH

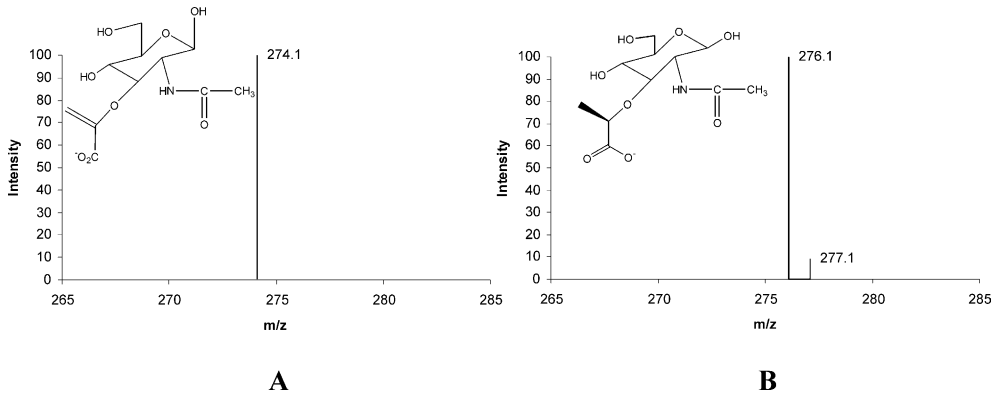

Figure 5. (A) Mass spectrum of purified UDP-*N*-acetylglucosamine-enolpyruvate eluted at 36 min on FPLC and chemical structure of the cleavage product *N*-acetylglucosamine-enolpyruvate. The major peak *m*/*z* 274.1 corresponds to the mass of the ionized *N*-acetylglucosamine-enolpyruvate after the gain of one proton. (B) Mass spectrum of purified UDP-*N*-acetylmuramic acid eluted at 32 min on FPLC and chemical structure of the cleavage product *N*-acetylmuramic acid. The major peak *m*/*z* 276.1 corresponds to the mass of the ionized *N*-acetylmuramic acid after the gain of one proton.

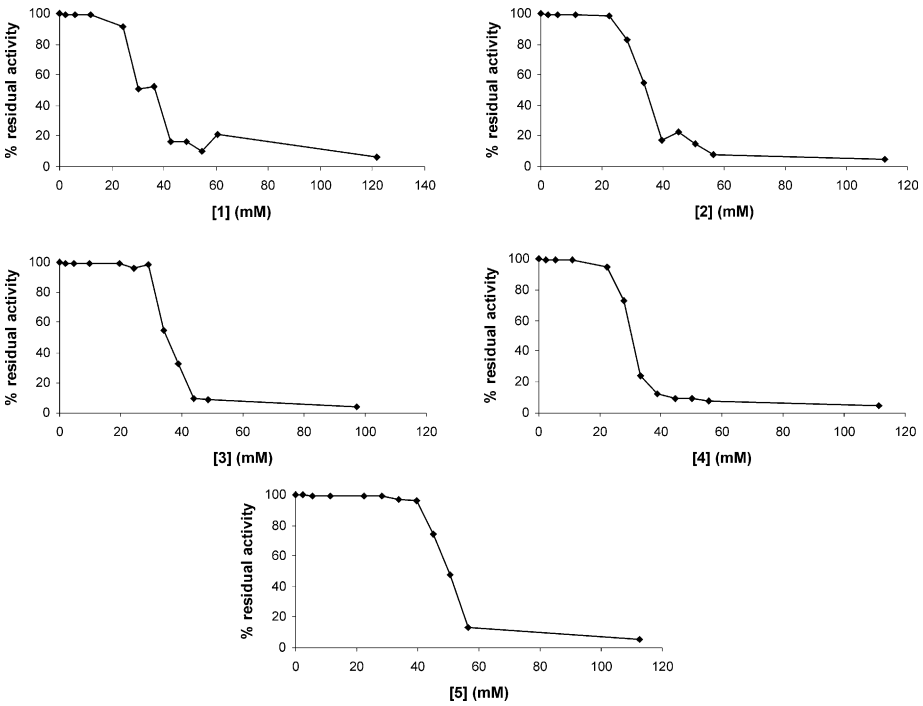

Figure 6. IC_{50} determinations for MurC inhibitors derived from combinatorial chemistry. The % of ATP [α-^{32}P] hydrolysis was measured as a function of the concentration of each compound.

absorbance at 340 nm. The reaction rates of both MurA and MurB remained unchanged in the presence of the 5 inhibitors (data not shown). This demonstrates that all of the 5 compounds are specific inhibitors of MurC. This specificity to MurC was expected since our libraries were designed as amino acid molecules bearing substituted side chains, which favours an interaction with enzymes possessing amino acid substrates.

Inhibition assays on MurC and IC_{50} determination

The 5 compounds were tested on the MurC catalyzed reaction. Inhibition of ATP hydrolysis was used to assess the inhibitory properties of the synthesized compounds. This assay depends on the hydrolysis of one molecule of ATP into ADP accompanying the L-alanine addition using α-^{32}P radioactively labelled ATP in the reaction mixture, then separating ATP from ADP by TLC. The efficiency of the inhibition of each compound was estimated from the decrease in % of ATP hydrolysis in the presence of different inhibitor concentrations. The % residual activity with various inhibitor concentrations was obtained by comparing the % ATP hydrolysis in presence of each concentration to the % ATP hydrolysis of the uninhibited MurC reaction (Fig. 6). The IC_{50}s obtained from the measurements were 30, 34, 35.2, 30.5, and 50.2 mM for **1**, **2**, **3**, **4**, and **5**, respectively.

Conclusion

We developed a rapid and reliable screening assay by TLC to test for inhibitors of the MurA,-B and-C enzymes. Several libraries of amino acid derivatives were synthesized by the mix-and-split combinatorial chemistry approach. The synthesized libraries were tested by the TLC assay and by agar diffusion assay on bacteria. Compounds in the active libraries were synthesized individually by parallel synthesis and retested by the TLC assay and by the MIC test on bacteria. We identified 5 potential lead compounds, which were shown to be inhibitors of the MurC enzyme. Investigations on the structure–activity relationships of the obtained data and on the structural features of the most active compounds would permit the design of more potent cell wall inhibitors.

Experimental

DNA manipulations, reagents and techniques

All reagents were purchased from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise indicated. Buffer D used to maintain enzymes was 20 mM potassium phosphate, 1 mM 2-mercaptoethanol, 0.1 mM $MgCl_2$, 15% (v/v) glycerol (pH 7.0). Restriction endonuclease and T4 ligase were obtained from New England Biolabs (Beverly, MA, USA). Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures. Recombinant plasmids containing *P. aeruginosa mur* genes were propagated in *E. coli* NovaBlue, *endA1 hsdR17*(r^-_{K12} m^+_{K12}) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA*$^+$*B*$^+$ *lacI*^{q}*Z* δ *M15*::Tn*10*], (Novagen, Madison, WI, USA) prior to protein synthesis in *E. coli* BL21, F$^-$ *ompT hsdS*$_B$(r^-_B m^-_B) *gal dcm* (DE3), (Novagen).

Cloning and overexpresion of *P. aeruginosa murA, -B* and *-C*

Polymerase chain reaction (PCR) cloning was used to obtain MurA, -B and -C proteins with a His-tag at their C-terminal.[21,22] Upper and lower primers were designed to contain appropriate restriction sites. Purified PCR products were digested with the restriction enzymes included in upper and lower primers and were cloned into the corresponding sites of the expression vector pET30a (Novagen) under the control of the bacteriophage T7 promoter. The recombinant plasmids pMON3005, pMON3006 and pMON3004 were introduced into the *E. coli* host strain BL21(λDE3) (Novagen) by electroporation for expression of MurA, -B and -C respectively, with His-Tag at their C-termini. Recombinant proteins were purified to homogeneity on an affinity nickel column as previously described.[21,22]

Screening assay for cell wall inhibitors

The assay contained, in a final volume of 20 μL, 50 mM bis–Tris Propane (pH 8.0), 1 mM UDP-*N*-acetyl glucosamine, 1 mM phosphoenol pyruvate, 1 mM β-NADPH, 10 mM L-alanine, 5 mM DTT, 5 mM $MgCl_2$ (Fisher Scientific, Fair Lawn, New Jersey, USA), 1 μL of purified MurA (0.6 mg/mL), 1 μL of purified MurB (2 mg/mL), 1 μL of purified MurC (1 mg/mL) and 1 μL of ATP [α-^{32}P] (3000 Ci/mmol, 10 mCi/mL) (PerkinElmer Life Sciences, Boston, Massachusetts, USA). Inhibitors were dissolved in DMSO (Laboratoire MAT, Beauport, Québec, Canada) to 20 μg/μL. Eight microliters of each inhibitor solution in DMSO were added to each reaction giving final inhibitor concentration of 8 μg/μL. Eight microliters of DMSO were added to the control reaction. The reactions were started by the addition of the three enzymes and ATP and then incubated at 37 °C for 60 min. Two microliters of each reaction were spotted on a 20×20 cm Baker-flex Cellulose PEI flexible sheet for TLC (J.T. Baker, Phillipsburg, NJ, USA). The chromatogram was developed in 0.75 M KH_2PO_4 (pH 3.4) for at least 3 h, then put in Saran wrap and exposed to a 20×40 cm Imaging plate (Fuji Photo Film, Kanagawa, Japan) for 60 s. The Imaging plate was scanned in a Fujix Bio-Imaging Analyzer model IPR1000 using the program Image Reader BAS-1000 Version 1.02 (Fuji Photo Film, Tokyo, Japan). The radioactive spots corresponding to ATP and ADP were quantified using the program Science Lab 2001 Image Gauge Version 4.0 (Fuji Photo Film, Tokyo, Japan).

Synthesis of libraries of molecules by combinatorial chemistry

Coupling of the central amino acid on oxime resin (R^1). Oxime resin with a loading of 0.5 mmol/g was introduced into a peptide synthesis ampoule. The resin was washed with DCM. The central amino acid (5 equiv) was dissolved in a DCM–DMF 1:1 mixture and the solution was cooled to 0 °C. DIC (5 equiv) was added and the solution was shaken for 30 min. The resulting suspension was poured into the ampoule and shaken for 24 h. The ampoule content was filtered by suction. The resin was successively shaken with the following solvents: 3×DMF, 3×methanol, 3×DMF and 3×methanol. After washing, the resin was extensively dried under vacuum. After this first coupling, the loading of resin was determined by Kaiser's quantitative colorimetric ninhydrine test.[27]

Acetylation of non-substituted sites. Non-substituted sites on the oxime resin were blocked by acetylation. The resin was washed with DMF. Acetic anhydride–DMF 1:1 mixture was added to the ampoule followed by DIEA (1 mL/5 g resin). The ampoule was shaken for 2 h. The ampoule content was filtered by suction and the resin was washed following the same sequence mentioned above and dried under vacuum.

Deprotection of the BOC group. The resin was washed with DCM. 2,2,2-Trifluoroacetic acid–DCM 1:1 mixture was added to the ampoule. After shaking for 30 min, the ampoule content was filtered by suction and the resin was washed following the same sequence mentioned above and dried under vacuum.

Coupling and modification of N-terminal (R^2). The resin was washed with DMF. The desired aromatic carboxylic acid R^2 (5 equiv) was dissolved in a DCM–DMF 1:1 mixture and the solution was cooled to 0° C. DIC (5 equiv) was added and the solution was shaken for 5 min. $HOBtH_2O$ (5 equiv) was added to this solution and the whole mixture was shaken for another 30 min. The suspension was then introduced into the ampoule, DIEA (1.5 equiv) was added and the ampoule content was shaken for 2 h. At the end of the coupling, the ampoule content was filtered by suction and the resin was washed following the same sequence mentioned above and dried under vacuum. Determination of coupling efficiency was done by Kaiser's qualitative colorimetric ninhydrine test.[28] In case of a negative test, this coupling step was repeated.

Cleavage and modification of C-terminal (R^3). With sodium hydroxide. The resin was washed with THF. A solution containing 0.1 N NaOH 10% in THF was then introduced into the ampoule. The ampoule was shaken for 4 h. At the end of the coupling, the ampoule content was filtered by suction and the resin was washed several times with DCM and methanol alternating the solvents at each wash. The filtrate was recovered and the solvents were removed under vacuum. The dried product was dissolved in glacial acetic acid, then lyophilized.

With propylamine.[29] The resin was washed with DCM. Propylamine (1.0 equiv) was introduced into the ampoule with a certain amount of DCM to allow efficient shaking. The solution was shaken for 1 h. At the end of the cleavage, the ampoule content was filtered by suction and the resin was washed several times with DCM and methanol alternating the solvents at each wash. The filtrate was recovered and the solvents were removed under vacuum. The dried product was dissolved in glacial acetic acid, then lyophilized.

With hydroxylamine.[30] The resin was washed with DCM. A hydroxylamine solution (1.0 equiv) was introduced into the ampoule with a certain amount of DCM to allow efficient shaking. The solution was shaken for 1 h. At the end of the cleavage, the ampoule content was filtered by suction and the resin was washed several times with DCM and methanol alternating the solvents at each wash. The filtrate was recovered and the solvents were removed under vacuum. The dried product was dissolved in glacial acetic acid, then lyophilized.

With (aminomethyl)-pyridines[29]. The resin was washed with DCM. The needed quantities of (aminomethyl)-pyridine substituted at position 2, 3 or 4 (1.0 equiv) were introduced into the ampoule with a certain amount of DCM to allow efficient shaking. The solution was shaken for 4 h. At the end of the cleavage, the ampoule content was filtered by suction and the resin was washed several times with DCM and methanol alternating the solvents at each wash. The filtrate was recovered and the solvents were removed under vacuum. The dried product was dissolved in glacial acetic acid, then lyophilized.

Agar diffusion assay. Cultures of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were grown in 5 mL Mueller Hinton broth (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA) until a cell density of $A_{600nm} = 0.5$ was reached. 100 mL aliquots of Mueller Hinton Agar (Becton Dickinson Microbiology Systems) were poured into 150 mm Petri dishes. Wells were made in the agar plates with sterile Pasteur pipettes. Sterile cotton tipped applicators were used to inoculate the surface of agar plates with the bacterial cultures. 100 μL of 20 μg/μL solution of inhibitors in DMSO were added to each well. Plates were incubated at 37 °C overnight without being inverted.

Drug susceptibility testing. MICs were determined for a panel of microorganisms according to standard procedures.[31] Bacterial cultures were inoculated in 96-well plates containing Mueller Hinton broth (Becton Dickinson Microbiology Systems) with various concentrations of the tested molecules. Growth was monitored by measuring the optical density of the culture after incubation at 37 °C for 24 h.

Synthesis and purification of UDP-*N*-acetylglucosamine-enolpyruvate. A reaction mixture containing TAPS (50 mM, pH 8.0), UDP-*N*-acetyl glucosamine (2.1 mM), phosphoenol pyruvate (3.9 mM), and DTT (5 mM) in a final volume of 5 mL was prepared in a 50 mL plastic tube. Fifty microliters of purified MurA (0.6 mg/mL) were added to initiate the reaction. The reaction was incubated for 5 h at 37 °C.[32] A control reaction was performed by adding all the substrates and omitting the enzyme. The reaction mixture was filtered through an Amicon YM 10 membrane (Millipore Corporation, Bedford, MA, USA) to remove the enzyme. Separation was performed by FPLC using ÄKTAexplorer (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). The filtered reaction was applied to a 10 μm particle size MonoQ HR 10/10 anion-exchange column (Amersham Pharmacia Biotech) at room temperature. Flow rate was 4 mL/min. UV detection was set at 254 nm. Lines A and B were 0.02 M NH_4OAc (pH 5.0) and 1.0 M NH_4OAc (pH 5.0), respectively. Injection volume was 4 mL. The following elution steps were used: an isocratic feed of A for 5 min, a first linear gradient from 0 to 20% B for 20 min, a second linear gradient from 20 to 65% B for 15 min, and finally a third linear gradient from 65 to 100% B for 5 min. The control was incubated for 5 h at 37 °C then analyzed by FPLC. Fractions of the peak corresponding to UDP-*N*-acetylglucosamine-enolpyruvate were pooled, concentrated and lyophilized. The lyophilized flocculent powder was dissolved in water then re-lyophilized three times to remove the ammonium acetate buffer.

Synthesis and purification of UDP-*N*-acetylmuramic acid. A reaction mixture containing TAPS (50 mM, pH 8.0), UDP-*N*-acetyl glucosamine (2.1 mM), phosphoenol pyruvate (3.9 mM), β-NADPH (2 mM) and DTT (5 mM) in a final volume of 5 mL was prepared in a 50 mL plastic tube and flushed with nitrogen for 30 min. Fifty microliters of purified MurA (0.6 mg/mL) and 12.5 μL of purified MurB (2 mg/mL) were added to initiate the reaction. The reaction was flushed with nitrogen for another 10 min and incubated for 5 h at 37 °C.[32] A control reaction was performed by adding all the substrates and omitting the enzymes. The reaction mixture was filtered through an Amicon YM 10 membrane (Millipore Corporation) to remove the enzymes. Separation was performed as described above. Fractions of the peak corresponding to UDP-*N*-acetylmuramic acid were pooled, concentrated and lyophilized. The lyophilized flocculent powder was dissolved in water then re-lyophilized three times to remove the ammonium acetate buffer.

Mass spectrometry. The product of each of the two reactions was analyzed on a quadrupole mass spectrometer detector (Agilent Technologies, Mississauga, ON, Canada, model HP 1100 LC-MSD). 1.0 M NH_4OAc (pH 5.0) was used as a mobile phase. Flow rate was 0.4 mL/min. Injection volume was 30 μL of the FPLC pooled fractions of the peak corresponding to the product. The electrospray ionization (ESI) was set at $V_{cap} = 4500$ V, nebulizing gas pressure = 35 psi, drying gas flow rate = 13 L/min, drying gas temperature = 350° C, with the quadrupole scanning from 150 to 850 m/z every 1.03 s with a step size of 0.15 amu. The system control and data evaluation were done on a HP ChemStation for LC/MS.

LC/MS system. The sample analysis was performed on a HPLC coupled to a mass spectrometer (LC/MS) (Agilent Technologies, model HP 1100 LC-MSD) comprised of a quaternary pump, a vacuum degasser, a refrigerated autosampler, a column compartment, a variable wavelength detector and an atmospheric pressure electrospray ionization (API-ES), quadrupole mass spectrometer detector (MSD). The system control and data evaluation were done on a HP ChemStation for LC/MS. Separation was done using a C_5 reversed-phase column 0.46×25 cm (Phenomenex, Torrance, CA, USA) at room temperature. Flow rate was 0.5 mL/min

and injection volume was 10 μL. A linear gradient from a 10:90 to 100:0 ACN-water (with 0.1% of TFA) over 45 min was used. Mass spectrometry detection was performed with the ESI set at $V_{cap} = 4500$ V, nebulizing gas pressure = 35 psi, drying gas flow rate = 13 l/min, drying gas temperature = 350 °C, with the quadrupole scanning from 50 to 500 m/z every 1.03 s with a step size of 0.15 amu.

NMR analysis. NMR experiments were done on a Bruker AC-F-300 MHz spectrometer using standard software. All measurements were made at 25 °C on a 5 mg sample dissolved in 0.6 mL of DMSO-d_6. The residual proton resonance of DMSO was used as an internal reference at 2.5 ppm for ^{1}H spectra.

Assay for MurA inhibition. Inhibition assay of MurA was done using the phosphate release assay. The assay contained, in a final volume of 200 μL, 25 mM Tris–HCl (pH 7.8), 10 mM UDP-*N*-acetylglucosamine, 10 mM PEP, and 20 μL of purified MurA (0.6 mg/mL). Inhibitors were dissolved in DMSO to 100 μg/μL. Twenty microliters of each inhibitor solution in DMSO were added to each reaction, giving final inhibitor concentration of 10 μg/μL. Twenty microliters of DMSO were added to the control reaction. The reactions were started by the addition of the MurA enzyme and then incubated at 37 °C. Twenty microlitre aliquots were removed at 0, 10, 20, 30, 40, 50, and 60 min and assayed for inorganic phosphate by the method of Lanzetta et al.[33] Lanzetta's malachite green-ammonium molybdate assay mix (1.6 mL) was added to each 20 μL aliquot. Inorganic phosphate was quantitated by measuring the optical A_{660}. Sterox detergent and citric acid were omitted from the method of Lanzetta et al.[34]

Assay for MurB inhibition. Inhibition assay of MurB was done by monitoring the oxidation of NADPH as a decrease in absorbance at 340 nm.[35] The assay contained, in a final volume of 1 mL, 50 mM bis–Tris–propane (pH 8.0), 50 mM KCl, 5 mM DTT, 150 μM NADPH, 1 mM UDP-*N*-acetylglucosamine-enolpyruvate, and 20 μL purified MurB (2 mg/mL). Inhibitors were dissolved in DMSO to 100 μg/μL. Eighty microliters of each inhibitor solution in DMSO were added to each reaction, giving final inhibitor concentration of 8 μg/μL. Eighty microlitres of DMSO were added to the control reaction. The reaction mixtures without NADPH and MurB were incubated at 37 °C for 10 min before NADPH was added. The reactions were started by the addition of the MurB enzyme. The decrease in NADPH absorbance at 340 nm (extinction coefficient = 6220 cm^{-1} M^{-1}) was monitored on a Cary 1 spectrophotometer. The reaction rates were calculated from the linear portion of the progress curve after addition of MurB.

Assay for MurC inhibition and IC_{50} determination. The assay contained, in a final volume of 20 μL, 50 mM bis–Tris Propane (pH 8.0), 2 mM UDP-*N*-acetyl muramic acid, 10 mM L-alanine, 5 mM DTT, 5 mM $MgCl_2$ (Fisher Scientific, Fair Lawn, New Jersey, USA), 1 μL of purified MurC (1 mg/mL), and 1 μL of ATP [α-^{32}P] (3000 Ci/mmol, 10 mCi/mL) (PerkinElmer Life Sciences). Inhibitors were dissolved in DMSO (Laboratoire MAT) to 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/μL. Eight microlitres of each inhibitor solution at different concentrations in DMSO were added to each reaction, giving final inhibitor concentrations of 20, 8, 4, 2, 0.8, 0.4, 0.2, 0.08, 0.04, and 0.02 μg/μL, respectively. Eight microlitres of DMSO were added to the control reaction. The reactions were started by the addition of MurC and ATP and then incubated at 37 °C for 60 min. Two microlitres of each reaction were spotted on a 20×20 cm Baker-flex Cellulose PEI flexible sheet for TLC (J.T.Baker). The chromatogram was developed in 0.75 M KH_2PO_4 (pH 3.4) for at least 3 h, then put in Saran wrap and exposed to a 20×40 cm Imaging plate (Fuji Photo Film) for 60 s. The Imaging plate was scanned in a Fujix Bio-Imaging Analyzer model IPR1000 using the program Image Reader BAS-1000 Version 1.02 (Fuji Photo Film). The radioactive spots corresponding to ATP and ADP were quantified using the program Science Lab 2001 Image Gauge Version 4.0 (Fuji Photo Film). The 50% inhibitory concentrations (IC_{50}s) for the five inhibitors were obtained by plotting the% residual activity as a function of increasing inhibitor concentration.

Acknowledgements

This work was supported by the Canadian Bacterial Diseases Network via the Canadian Centers of Excellence and by a FQRNT-FRSQ infrastructure team grant to Roger C. Levesque. R.C.L. is a scholar of exceptional merit from Le Fonds de Recherche en Santé du Québec, A.E.Z. obtained a studentship from Univ. Laval and from La Fondation Marc Bourgie, M.B. was a CREFSIP scholar. We thank Le Service de protéomique de l'Est du Québec for technical assistance.

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