Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4588–4591

Synthesis of 4-phenoxybenzamide adenine dinucleotide as NAD analogue with inhibitory activity against enoyl-ACP reductase (InhA) of *Mycobacterium tuberculosis*

Laurent Bonnac,[a] Guang-Yao Gao,[a] Liqiang Chen,[a] Krzysztof Felczak,[a] Eric M. Bennett,[a] Hua Xu,[b] TaeSoo Kim,[b] Nina Liu,[b] HyeWon Oh,[b] Peter J. Tonge[b] and Krzysztof W. Pankiewicz[a,*]

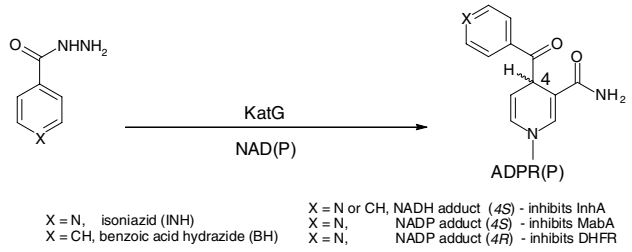
[a]*Center for Drug Design, University of Minnesota, Minneapolis, MN 55455, USA*
[b]*Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400, USA*

Received 11 April 2007; revised 23 May 2007; accepted 25 May 2007
Available online 31 May 2007

Abstract—The chemical synthesis of 4-phenoxybenzamide adenine dinucleotide (**3**), a NAD analogue which mimics isoniazid-NAD adduct and inhibits *Mycobacterium tuberculosis* NAD-dependent enoyl-ACP reductase (InhA), is reported. The 4-phenoxy benzamide riboside (**1**) has been prepared as a key intermediate, converted into its 5′-mononucleotide (**2**), and coupled with AMP imidazolide to give the desired NAD analogue **3**. It inhibits InhA with IC_{50} = 27 μM.

Tuberculosis (TB) is a chronic infectious disease caused by mycobacteria of the 'tuberculosis complex', including *Mycobacterium bovis, Mycobacterium africanum*, and mainly *Mycobacterium tuberculosis*. TB now kills more adults than all other infectious diseases combined. Active TB is usually treated with isoniazid in association with one or more other anti-TB drugs but multi-drug resistant TB (MDR-TB) and very recently extensively drug resistant TB (XDR-TB) has become a serious and unsolved public health problem.[1–6]

Isoniazid (isonicotinic acid hydrazide, INH) discovered in 1952 is still the most important drug for treatment of TB. It is a prodrug which requires metabolic oxidation by the *M. tuberculosis* enzyme, catalase-peroxidase katG,[4,7,8] to an isonicotinoyl radical which binds covalently to the position 4 of NAD(P) cofactor (Scheme 1).[8–10] The INH-NAD(P) adducts inhibit two enzymes involved in the fatty acid biosynthetic pathway of *M. tuberculosis*,[11–14] NAD-dependent enoyl-acyl carrier protein reductase (enoyl-ACP reductase, InhA) and



Scheme 1. Inhibition of *M. tuberculosis* reductases.

NAD(P)-dependent β-keto-ACP reductase (mycolic acid biosynthesis A, MabA).[15] Addition of the isonicotinoyl radical to the C4 of the nicotinamide ring can result in two stereoisomers, however, only 4(*S*) isomers of the INH-NAD and INH-NAD(P) are potent inhibitors of InhA (K_i = 0.75 nM) and MabA (K_i = 2.2 μM). In contrast, the 4(*R*) isomer INH-NAD(P) inhibits *M. tuberculosis* dihydrofolate reductase (DHFR), which is essential for nucleic acid synthesis (Scheme 1).[16] Interestingly, it was found that benzoic acid hydrazide is also metabolized by *M. tuberculosis* in a similar manner as INH and shows a potent inhibition of InhA.

A high prevalence of resistance to INH was observed, mainly due to emerging KatG mutants that do not acti-

Keywords: *M. tuberculosis*; Enoyl-ACP reductase (InhA); NAD analogues; Isoniazid; Fragment-based drug discovery.
* Corresponding author. Tel.: +1 612 625 7968; fax: +1 612 625 8252; e-mail: panki001@umn.edu

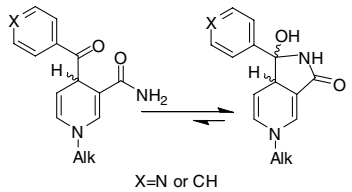
0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.05.084

vate or poorly activate INH. Therefore, it has been suggested that compounds that inhibit final targets of INH but do not require activation by KatG have tremendous promise as novel drugs for combating MDR-TB.[8,17] Preformed INH-NAD would be such a compound, but as dinucleotide it is useless as a potential drug due to metabolic instability and lack of cell permeability. Nevertheless, several reports have been recently published on chemical formation of the INH-NAD adduct (X = N), its benzoyl analogue (X = CH), and on synthesis of fragments of these important activated metabolites.[15,18–22] The studies of chemical activation of a mixture of INH and NAD revealed a rather complicated activation pathway in which two diastereomers [the 4(*S*)-an inhibitor and the 4(*R*)-inactive isomer)] were formed together with four cyclic diastereoisomers (hemiamidals).[19,20]

Chemical synthesis of fragments of the NAD adducts (Scheme 2) supplemented earlier observation on poor stability of INH-NAD adduct and formation of hemiamidals. However, none of the synthesized 4-substituted nicotinamide fragments showed activity against InhA.[21,22]

Chemical activation of a mixture of INH and NAD resulted in the formation of INH-NAD adduct but the cleavage of the unstable glycosidic bond of nicotinamide riboside (NR) moiety of NAD was observed resulting in release of ADP ribose.[18] In addition, NAD^+ is a substrate in numerous biochemical processes[23] in which the glycosidic bond is cleaved. Thus, the key issue in designing a drug, based on inhibition of InhA, is the construction of a tight binding NAD mimic, which *will be metabolically stable.*

To address undesirable hemiamidal formation, we redesigned the attachment at the position 4, and began by investigating the geometry of the position 4 using molecular modeling.[24] Docking reproduced the NAD binding mode observed in the X-ray structure (Fig. 1A) with an RMS error of 0.34 Å and the INH-NADH adduct with an RMS error of 0.80 Å (Fig. 1B). With NADH adducts, the stereochemistry at position 4 is critical. Modeling shows that changing from *S* to *R* causes the INH ring to overlap with the backbone of residues 193–194. In the docking simulation, these bad contacts forced the nicotinamide portion of the molecule out of its normal binding pocket for all conformations found. The global minimum had an RMSd of 6.6 Å from the X-ray structure, despite the adenosine half of the molecule remaining in the correct position (Fig. 1C).



Scheme 2. Stereochemistry and equilibrium of fragments of INH-NAD adducts.

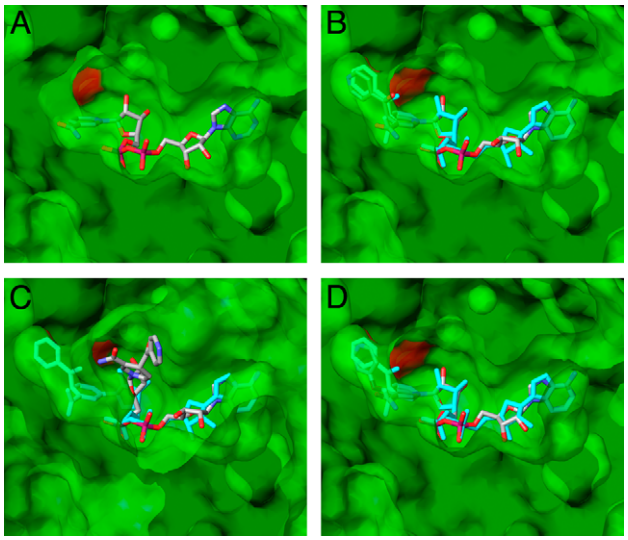


Figure 1. Binding of NAD and NAD adducts to InhA. (A) Cocrystal structure with NAD.[25] (B) Docked INH-NAD adduct (colored by atom) superimposed with the INH-NAD X-ray structure (blue) 9. (C) Docked (4*R*) INH-NAD superimposed with the (4*S*) X-ray structure (blue). (D) Docked 4-phenoxy NAD superimposed with the INH-NAD X-ray structure (blue). For all panels, a cutaway of the protein surface is shown in green, except for the surface of Phe149, which moved to accommodate the adducts and is shown in red. Docking results are the lowest energy conformations found.

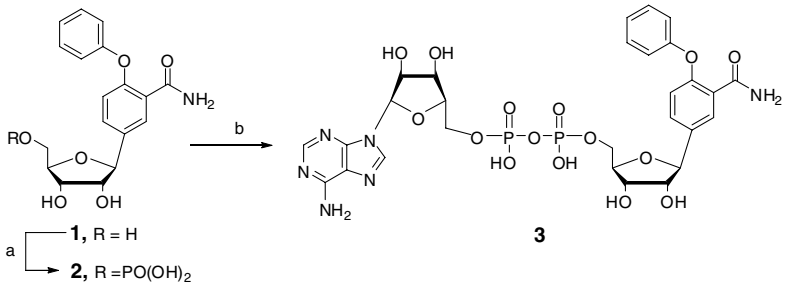
In contrast, a 4-phenoxy substitution of NAD, where the position 4 is sp^2-hybridized, resulted in a global minimum with a low 0.69 Å RMSd compared to the INH-NADH adduct (Fig. 1D).

To address glycosidic bond instability, we also replaced the nicotinamide ring nitrogen with carbon, a change which by itself gives benzamide adenine dinucleotide (BAD or 1-deaza-NAD).[26,27] Thus, we focused on synthesis of 4-phenoxy-substituted BAD analogue **3**.

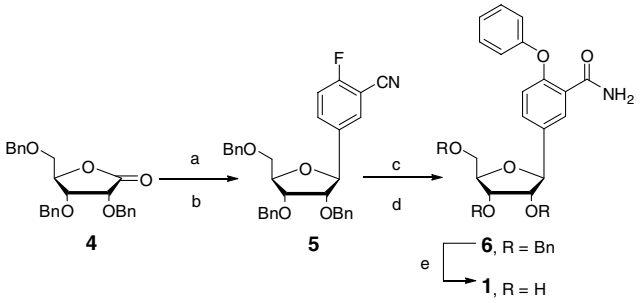
The synthesis of **3** (Scheme 3) was accomplished by phosphorylation of **1** with $P(O)Cl_3$ (path a) and coupling of the 5′-monophosphate **2** with adenosine-5′-monophosphate imidazolide (path b).[28]

The key intermediate for the above synthesis is a novel C-nucleoside **1**, which we synthesized *via* stereo-specific coupling of the Grignard reagent (prepared from commercially available 2-fluoro-5-iodobenzo-nitrile) with the protected ribonolactone **4** (Scheme 4) followed by reduction. The fluorinated carbon in the activated *ortho* position of the nitrile was easily substituted by a phenolate anion generated from phenol under basic condition.[29] Then, the nitrile group was hydrolyzed to the carboxyamido group with potassium trimethylsilanolate to give **6**.[30] The protective groups of the ribose are finally removed with boron tribromide to give the desired **1**. The structures of all new compounds **1-3** were confirmed by ^1H, ^{31}P NMR and HRMS, and their purity was established by HPLC.[31]

Compounds **1**–**3** were evaluated against InhA. The inhibition was monitored by following the inactivation of



Scheme 3. Synthesis of 4-phenoxybenzamide adenine dinucleotide (**3**).



Scheme 4. Efficient, stereoselective synthesis of a key C-nucleoside **1**. Reagents: (a) *i*-PrMgCl, 2-fluoro-5-iodobenzonitrile, THF; (b) BF_3OEt_2, Et_3SiH; (c) Phenol, K_2CO_3; (d) Me_3SiOK, THF; (e) BBr_3, CH_2Cl_2.

InhA in reaction mixtures containing NADH, dodecenoyl CoA, the inhibitor, and InhA.[32]

The C-nucleoside **1** showed weak activity at 700 μM. However, the monophosphate **2** showed 12% inhibition at concentration of 100 μM. The BAD analogue **3** inhibited InhA with an IC_{50} of 27 μM (Table 1), confirming our initial hypothesis that the simple aromatic mimics of the INH-NAD adduct would show inhibitory activity against InhA. Although the BAD analogue **3** is a micromolar inhibitor it does not require metabolic activation and did not form a complicated mixture of active and inactive stereo-isomers. If phosphorylated to the corresponding NAD(P) analogues, **3** could inhibit two other *M. tuberculosis* enzymes, MabA and DHFR. Interestingly, we recently found that benzamide adenine dinucleotide (BAD) is a substrate for bacterial NAD-kinase.[28]

The results indicate that further searching for more potent inhibitors of InhA based on a similar approach is justified. Once a nanomolar inhibitor with aromatic structure similar to that of **3** is found, a simple replacement of the pyrophosphate oxygen by the CH_2 group will afford the corresponding bis(phosphonate) analogue of INH-NAD with expected improved metabolic stability and drug-like properties.[33,34]

Table 1. Inhibition of InhA by compounds **1**–**3**

Compounds	InhA inhibition
4-PhenoxyBR (**1**)	25% Inhibition at 700 μM
4-PhenoxyBRMP (**2**)	12% Inhibition at 100 μM
4-PhenoxyBAD (**3**)	IC50 = 27 μM

Finally, in contrast to other reports we found that much smaller fragments of our target molecule **3**, such as nucleoside **1** and mono-nucleotide **2**, showed 25% and 12% of inhibition of InhA at concentration of 700 μM and 100 μM, respectively. This inspired us to synthesize a number 4-substituted aromatic C-nucleoside and carbocyclic nucleoside analogues. Indeed, some of these compounds (*at the nucleoside level*) showed 50% or more inhibition of InhA at 100 μM. According to the principles of fragmented-based drug design we expect to boost their activity by converting them into the corresponding NAD analogues.

Acknowledgments

These studies were funded by the Center for Drug Design, University of Minnesota. We thank the Minnesota Supercomputing Institute for computer time.

References and notes

1. Aziz, M. A.; Wright, A.; Laszlo, A.; De Muynck, A.; Portaels, F.; Van Deun, A.; Wells, C.; Nunn, P.; Blanc, L.; Raviglione, M. *Lancet* **2006**, *368*, 2142.
2. Raviglione, M. C.; Smith, I. M. *N. Engl. J. Med.* **2007**, *356*, 656.
3. Pasqualoto, K. F.; Ferreira, E. I.; Santos-Filho, O. A.; Hopfinger, A. J. *J. Med. Chem.* **2004**, *47*, 3755.
4. Wei, C. J.; Lei, B.; Musser, J. M.; Tu, S. C. *Antimicrob. Agents Chemother.* **2003**, *47*, 670.
5. Morlock, G. P.; Metchock, B.; Sikes, D.; Crawford, J. T.; Cooksey, R. C. *Antimicrob. Agents Chemother.* **2003**, *47*, 3799.
6. Ormerod, L. P. *Br. Med. Bull.* **2005**, *73*, 17.
7. Zhao, X.; Yu, S.; Magliozzo, R. S. *Biochemistry* **2007**, *46*, 3161.
8. Rawat, R.; Whitty, A.; Tonge, P. J. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13881.
9. Rozwarski, D. A.; Grant, G. A.; Barton, D. H.; Jacobs, W. R., Jr.; Sacchettini, J. C. *Science* **1998**, *279*, 98.
10. Lei, B.; Wei, C. J.; Tu, S. C. *J. Biol. Chem.* **2000**, *275*, 2520.
11. Schaeffer, M. L.; Agnihotri, G.; Volker, C.; Kallender, H.; Brennan, P. J.; Lonsdale, J. T. *J. Biol. Chem.* **2001**, *276*, 47029.
12. Kuo, M. R.; Morbidoni, H. R.; Alland, D.; Sneddon, S. F.; Gourlie, B. B.; Staveski, M. M.; Leonard, M.; Gregory, J. S.; Janjigian, A. D.; Yee, C.; Kreiswirth, B.; Iwamoto, H.; Perozzo, R.; Jacobs, W. R., Jr.; Sacchettini, J. C.; Fidock, D. A. *J. Biol. Chem.* **2003**, *26*, 26.

13. Choi, K. H.; Kremer, L.; Besra, G. S.; Rock, C. O. *J. Biol. Chem.* **2000**, *275*, 28201.
14. Kremer, L.; Douglas, J. D.; Baulard, A. R.; Guy, M. R.; Morehouse, C.; Alland, D.; Dover, L. G.; Lakey, J. H.; Jacobs, W. R., Jr.; Brennan, P. J.; Minnikin, D. E.; Besra, G. S. *J. Biol. Chem.* **2000**, *275*, 16857.
15. Ducasse-Cabanot, S.; Cohen-Gonsaud, M.; Marrakchi, H.; Nguyen, M.; Zerbib, D.; Bernadou, J.; Daffe, M.; Labesse, G.; Quemard, A. *Antimicrob. Agents Chemother.* **2004**, *48*, 242.
16. Argyrou, A.; Vetting, M. W.; Aladegbami, B.; Blanchard, J. S. *Nat. Struct. Mol. Biol.* **2006**, *13*, 408.
17. Basso, L. A.; Zheng, R.; Musser, J. M.; Jacobs, W. R., Jr.; Blanchard, J. S. *J. Infect. Dis.* **1998**, *178*, 769.
18. Nguyen, M.; Claparols, C.; Bernadou, J.; Meunier, B. *Chembiochem* **2001**, *2*, 877.
19. Nguyen, M.; Quemard, A.; Broussy, S.; Bernadou, J.; Meunier, B. *Antimicrob. Agents Chemother.* **2002**, *46*, 2137.
20. Broussy, S.; Coppel, Y.; Nguyen, M.; Bernadou, J.; Meunier, B. *Chemistry* **2003**, *9*, 2034.
21. Broussy, S.; Bernardes-Genisson, V.; Quemard, A.; Meunier, B.; Bernadou, J. *J. Org. Chem.* **2005**, *70*, 10502.
22. Delaine, T.; Bernardes-Genisson, V.; Meunier, B.; Bernadou, J. *J. Org. Chem.* **2007**, *72*, 675.
23. Such as mono- and poly-ADP-ribosylation of protein, cell signaling ((cyclic ADP-ribose), DNA repair and recombination, and histone deacetylation catalyzed by the Sir2 family of NAD-dependent deacetylases.
24. Modeling was based on the X-ray structure of the InhA/NAD complex (PDB entry 1ENY). The OPLS-2005 force field and the GB/SA solvation model, as implemented in MacroModel 9.1 (Schrodinger, Inc., 2006), were used for all simulations. Hydrogens were added and minimized, then the entire protein was minimized subject to a 10 kcal/mol-Å^2 restraint. This model was used for validation docking of NAD. When binding to INH-NADH adducts, Phe149 changes position to accommodate the adduct. The protein was frozen except for residues 149, 150, and 158, and a torsion search was performed to identify a low-energy conformation of Phe149 similar to that seen in the adduct X-ray structure (PDB entry 1ZID). This model was used for docking adducts, with Phe149 and the adduct unrestrained and the remainder of the protein frozen. MCMM/LMCS conformational searching of adducts was performed in blocks of 10,000 steps until (a) no new structures within 5 kcal/mol of the global minimum were found and (b) the rate of finding new structures within 10 kcal/mol of the global minimum decreased at least 10-fold relative to the first block of 10,000 steps. All searches converged after 40,000 or fewer steps. Minimizations were performed to a gradient of 0.05 kJ/mol-Å. RMS errors for the adducts were determined after superimposing PDB entry 1ZID on the minimized model. The RMS error for **3** was determined without considering the carbonyl oxygen of the INH/NAD adduct, since this atom has no counterpart in **3**.
25. Dessen, A.; Quemard, A.; Blanchard, J. S.; Jacobs, W. R., Jr.; Sacchettini, J. C. *Science* **1995**, *267*, 1638.
26. Zatorski, A.; Watanabe, K. A.; Carr, S. F.; Goldstein, B. M.; Pankiewicz, K. W. *J. Med. Chem.* **1996**, *39*, 2422.
27. Pankiewicz, K. W.; Lesiak, K.; Zatorski, A.; Goldstein, B. M.; Carr, S. F.; Sochacki, M.; Majumdar, A.; Seidman, M.; Watanabe, K. A. *J. Med. Chem.* **1997**, *40*, 1287.
28. Bonnac, L.; Chen, L.; Pathak, R.; Gao, G.; Ming, Q.; Bennett, E.; Felczak, K.; Kullberg, M.; Patterson, S. E.; Mazzola, F.; Magni, G.; Pankiewicz, K. W. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1512.
29. Kristensen, J. L.; Vedso, P.; Begtrup, M. *J. Org. Chem.* **2003**, *68*, 4091.
30. Merchant, K. J. *Tetrahedron Lett.* **2000**, *41*, 3747.
31. Compound **1**, ^{1}H NMR (δ, CD_3OD), 7.99 (H2, s, 1H), 7.56 (H4, d, J = 8.4 Hz, 1H), 7.39 [H3 (OPh), d, J = 7.8 Hz, 2H], 7.18 [H4 (OPh), t, J = 7.8, 1H], 7.04 [H2 (OPh), d, J = 7.8 Hz, 2H], 6.97 (H5, d, J = 8.4 Hz, 1H), 4.71 (H1′, d, J = 6.6 Hz, 1H), 4.0 (H2′, m, 1H), 3.98 (H4′, m, 1H), 3.85 (H3′, m, 1H), 3.75 (H5′5″, m, 2H). HRMS calcd for $C_{18}H_{20}NO_6$ $[M+H]^+$ 346.1212 found 346.1192.; Compound **3**, ^1H NMR (δ, D_2O), 8.31 (H8, Ade, s, 1H), 8.21 (H2, Ade, s, 1H), 8.04 (H2, s, 1H), 7.30 (H4, d, J = 8.4 Hz, 1H), 7.25 (H3(OPh), d, J = 7.8 Hz, 2H), 7.05 (H4(OPh), t, J = 7.8, 1H), 6.85 (H2 (OPh), d, J = 7.8 Hz, 2H), 6.66 (H5, d, J = 8.4 Hz, 1H), 5.87 (H1′, d, J = 5.4 Hz, 1H), 4.57 (H1′, d, J = 7.2 Hz, 1H), 4.44 (ribose, m, 1H), 4.29 (ribose, m, 1H), 4.22–3.88 (ribose, m, 8H). ^{31}P NMR (D_2O) −10.18, −9.98 (d, J= 21.14). HRMS calcd for $C_{28}H_{31}N_6O_{15}P_2$ $[M-H]^-$ 753.1401, found 753.1388.
32. All kinetic experiments were carried out on a Cary 300 Bio (Varian) spectrophotometer at 25 °C. A typical reaction mixture contained 30 mM PIPES, 150 mM NaCl, pH 8.0, 100 nM InhA, 25 μM dodecenoyl CoA, 250 μM NADH, and a certain concentration of the inhibitor. The reaction was initiated by the addition of InhA, and then monitored at 340 nm. The initial velocities were measured from the linear period of the assays (usually the first 1/2 min). The inhibitions of InhA by compounds **1** and **2** were tested at the specified concentration. For compound **3**, inhibition assays were carried out at different inhibitor concentration ranging from 0 to 200 μM, and then the data were fitted into the following equation to obtain the IC_{50} value: $v = v_0/(1 + [I]/IC_{50})$.
33. Pankiewicz, K. W.; Lesiak-Watanabe, K. B.; Watanabe, K. A.; Patterson, S. E.; Jayaram, H. N.; Yalowitz, J. A.; Miller, M. D.; Seidman, M.; Majumdar, A.; Prehna, G.; Goldstein, B. M. *J. Med. Chem.* **2002**, *45*, 703.
34. Pankiewicz, K. W.; Patterson, S. E.; Black, P. L.; Jayaram, H. N.; Risal, D.; Goldstein, B. M.; Stuyver, L. J.; Schinazi, R. F. *Curr. Med. Chem.* **2004**, *11*, 887.