THE TRIFLUOROACETYLPYRIDINE ANALOG OF NICOTINAMIDE ADENINE DINUCLEOTIDE+

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+Dedicated to Alan R. Katritzky

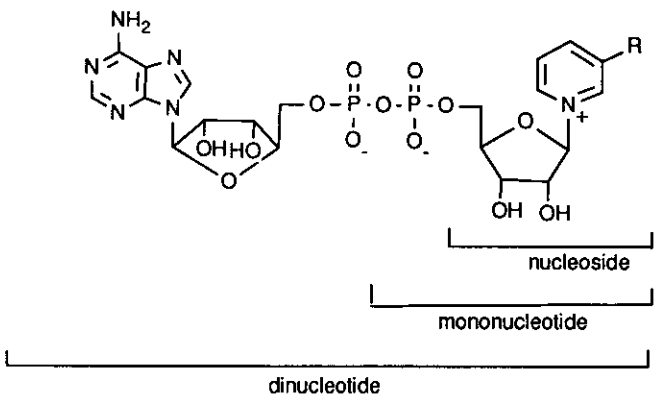
Abstract- 3-Trifluoroacetylpyridine adenine dinucleotide has been synthesized enzymically in two steps. The first step involved forming a glycosidic link via the ring nitrogen of 3-trifluoroacetylpyridine to ribose-5-phosphate by reaction of 3-trifluoroacetylpyridine with 5-phosphoribosyl pyrophosphate, catalyzed by nicotinamide phosphoribosyl transferase. The resulting mononucleotide was allowed to react with adenosine triphosphate in the presence of NAD pyrophosphorylase to yield the NAD analog, trifluoroacetylpyridine adenine dinucleotide. The analog behaved as an inhibitor of several NAD-dependent dehydrogenases, but it did not act as a substrate.

Analogs of natural metabolic intermediates, in which one or more hydrogen or oxygen atoms have been replaced by fluorine atoms, have proved to be useful probes for metabolic studies and for investigating the binding of ligands to enzymes and other proteins.[1] A particular feature of fluorinated analogs is that the chemical shift of the ^{19}F nmr spectrum, with its high sensitivity to dielectric constant and pH, and its position far removed from the proton signals of the protein, can serve as a reporter group for the microenvironment of the binding site.[2]

In view of the central metabolic role of NAD (**1b**) in reactions catalyzed by oxidoreductases, a fluorinated analog of NAD would be expected to be a useful probe. The work of Kaplan and coworkers[3] has shown that the 3-substituent on the pyridine ring of NAD can often be altered without adversely affecting the ability of the analog to bind to NAD-dependent enzymes. Indeed in some cases, e.g. 3-acetylpyridine adenine dinucleotide, even substrate activity is retained. The 3-trifluoroacetyl group might be expected to be an especially useful substituent in view of the strong singlet in the ^{19}F nmr spectrum. We now report the synthesis of trifluoroacetylpyridine adenine dinucleotide (**1a**).

Attempts to synthesize **1a** chemically met with only partial success. The reaction of 3-trifluoroacetylpyridine (prepared from 3-bromopyridine[4]) with 1-chloro-2,3,5-tribenzoyl-D-ribofuranose, using the method of Haynes et al.,[5] proceeded smoothly to give the corresponding tribenzoylated derivative of the nucleoside (**1a**).

Debenzoylation (NH_3, MeOH) gave nucleoside (**1a**) as a pale yellow hygroscopic powder, identified by its ir and ^1H nmr spectra. The 100 MHz ^{19}F spectrum was a singlet 16.1 ppm downfield (CF_3 COOH). However the yield of this product (8%) was too low to be of practical use, and the chemical route was abandoned. Other debenzoylation methods (NaOMe; MeOH, HCl) resulted in loss of fluorine.



1a, R=$COCF_3$
1b, R=$CONH_2$

NAD transglycosidase catalyzes the *N*-glycosidic exchange of nicotinamide with other pyridine derivatives on the dinucleotide (**1b**), and many NAD analogues have been prepared using this enzyme. 3-Trifluoroacetylpyridine was not accepted as a substrate by this enzyme, although it was found to inhibit the enzyme in its catalysis of the exchange of nicotinamide and 3-acetylpyridine on NAD.

Successful synthesis was achieved through a two-step enzymic method. Nicotinamide phosphoribosyl transferase from Lactobacillus fructosus catalyzed the formation of mononucleotide (**1a**) from 5-phosphoribosyl-1-pyrophosphate and 3-trifluoroacetylpyridine. On tlc using CM-cellulose (n-butanol:methanol:water:0.88 ammonia::60:20:20:1) mononucleotide (**1a**) had an identical Rf (0.80) to that of authentic mononucleotide (**1b**). The ^{19}F nmr signal was a singlet 8.70 ppm upfield (CF_3C00H). Mononucleotide (**1a**) was converted to dinucleotide (**1a**) by reaction with adenosine triphosphate catalyzed by NAD pyrophosphorylase. Inorganic pyrophosphatase was included to catalyze the hydrolysis of released pyrophosphate and drive the reaction to completion. Tlc of the product, dinucleotide (**1a**), on CM-cellulose (1M acetic acid) and on PEI-cellulose (1.6M LiCl) gave Rf values (0.40 and 0.57 respectively) identical to those shown by NAD (**1b**). However, dinucleotide (**1a**) was more conveniently prepared by running the two enzymic steps in a combined reaction. By this procedure, a 72% yield of dinucleotide (**1a**) was obtained, based on 3-trifluoroacetylpyridine: ^{19}F Nmr, singlet 7.28 ppm upfield (CF_3COOH); λmax, 259 nm (ε=17.5 x 10^3). Both nucleotides were analyzed for phosphate,

adenylic acid, 3-trifluoroacetylpyridine and ribose content (Table 1). Ribose, phosphate and 3-trifluoroacetylpyridine were liberated by hydrolysis of **1a** with 2M NaOH. 3-Trifluoroacetylpyridine was estimated photometrically as its 2,4-dinitrophenylhydrazone by comparison with an authentic sample; λ_{max}, ($CHCl_3$) 375 nm (ε=3.0 x 10^3). Ribose was determined by using the orcinol procedure[6] and total phosphate by the method of Bartlett.[7] Adenylic acid, liberated from the analog with NAD pyrophosphatase,[8] was determined by using 5'-adenylic acid deaminase.[9]

Table 1 Analytical Data on Trifluroacetylpyridine Nucleotides

Component	Mononucleotide	Dinucleotide
3-trifluoroacetylpyridine	0.89 (1)	0.94 (1)
Ribose	1.05 (1)	1.95 (2)
Phosphate	0.93 (1)	1.92 (2)
Adenylic acid	0 (0)	1.03(1)

Results are given as moles per mole of analog.
Theoretical values in parenthesis.

Dinucleotide(**1a**)did not act as a cosubstrate for either lactate (pig muscle), alcohol (yeast), malate (pig heart mitochondria) or glyceraldehyde-3-phosphate (yeast) dehydrogenases, nor did it form an adduct with cyanide. Kaplan et al.[3] have observed that the pyridine substituents of NAD analogs able to act as a cosubstrate have a C=O or C=S directly attached to the pyridine ring at the 3-position. The failure of dinucleotide(**1a**)to act as a cosubstrate and its lack of reactivity with cyanide may be ascribed to the existence of the carbonyl group in aqueous solutions wholly as the gem diol, as has been found with 3-trifluoroacetylpyridine itself.[4]

Dinucleotide (**1a**) did act as an inhibitor for all four dehydrogenases. From the fractional inhibition observed, and assuming that **1a** acts as a competitive inhibitor with respect to the coenzyme, the following estimates of inhibition constants were calculated: lactate dehydrogenase, 0.18 mM; glyceraldehyde-3-phosphate dehydrogenase, 0.24 mM; alcohol dehydrogenase, 0.32 mM; malate dehydrogenase, 0.30 mM. These values are all within the experimentally detectable range of concentrations for ^{19}F nmr spectrometry, indicating the potential value of the analog.

Optimised preparation of dinucleotide 1a: 3-Trifluoroacetylpyridine (8.8 mg, 50 μmol) was incubated in 10 ml of 0.1M tris-acetate buffer, pH 8.2 containing $MgCl_2$ (23.8 mg, 0.25 mmol), disodium adenosine triphosphate (82.7 mg, 150 μmol), sodium 5-phosphoribosylpyrophosphate (20.6 mg, 50 μmol) nicotinamide phosphoribosyltransferase[10] (3 units), NAD pyrophosphorylase (from Sigma) (3 units) and inorganic pyrophosphatase (from Sigma) (500 units) at 37°C for 4 h. After deproteinization by addition of 10 ml of

ethanol, the mixture was lyophilized. The residue was dissolved in 0.1M potassium phosphate buffer pH 7.0 (10 ml), incubated with alkaline phosphatase (5 units) at 37°C for 15 min, and then chromatographed on Dowex 1x 8-acetate (5 cm x 2.5 cm dia.). After washing with water (150 ml), the column was eluted with 1M acetic acid (150 ml), and the eluates lyophilized to give dinucleotide (**1a**) as a white amorphous powder.

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