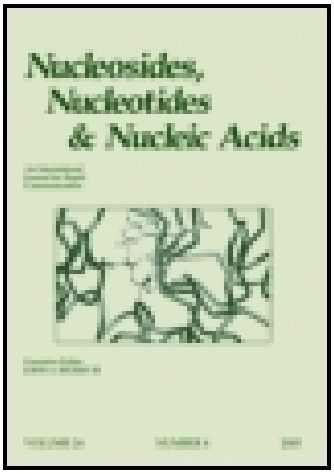

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Ashty Saleh [a] , Frans Compernolle [a] & Gerard Janssen [b]
[a] Laboratorium voor Organische Synthese , K. U. Leuven Celestijnenlaan 200F , B-3001, Leuven-Heverlee, Belgium
[b] Rega Instituut , Minderbroedersstraat 10, B-3000, Leuven, Belgium
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CHEMISTRY

AN IMPROVED SYNTHESIS OF N^6-(6-AMINOHEXYL)FAD

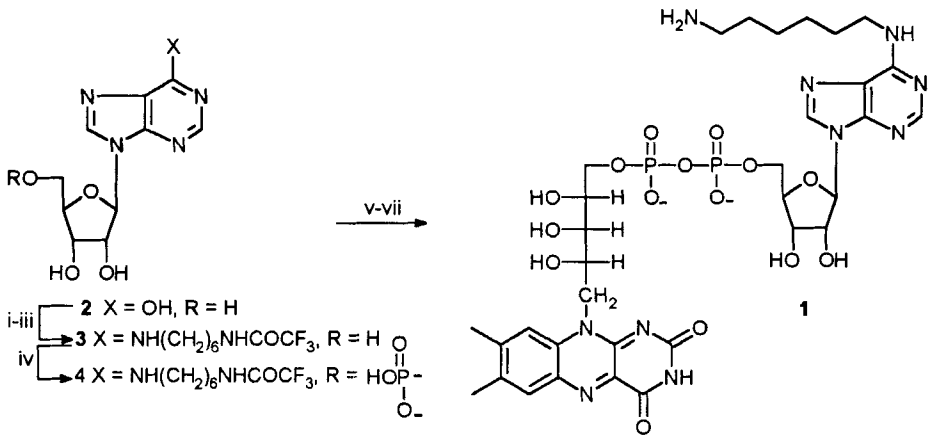
Ashty Saleh, Frans Compernolle*, and Gerard Janssen#
Laboratorium voor Organische Synthese, K.U. Leuven
Celestijnenlaan 200F, B-3001 Leuven-Heverlee, Belgium
and #Rega Instituut, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Abstract-We report an improved synthesis of N^6-(6-aminohexyl)FAD (**1**) using an efficient one-pot conversion of inosine to the *N*-trifluoroacetyl protected N^6-(6-aminohexyl)adenosine **3**. The 5'-*O*-phosphorylated AMP derivative **4**, activated as the imidazolide, was coupled with commercial sodium riboflavin phosphate by using 18-crown-6 in DMF.

Attachment of a ligand to the 6-aminohexyl side chain of the title compound **1** affords an FAD-labelled conjugate that can be used to measure the concentration of the free ligand in a competitive binding immunoassay.[1] When combined with apoglucose oxidase, the FAD conjugate serves as a prosthetic group for generation of glucose oxidase activity. The activity of the conjugate is strongly inhibited by antibody specific to the ligand and, hence, can be related to the concentration of free ligand competing for the antibody.

Our synthetic route to compound **1** (Scheme 1) differs from the published procedure[1,2] by the efficient one-pot conversion of inosine **2** to the *N*-trifluoroacetyl protected 6-aminohexyl derivative **3**. The 1,6-diaminohexyl side chain was introduced *via* the method of Vorbrüggen,[3] proceeding through silylation and acid catalysed substitution of the 6-trimethylsilyloxy group. Following removal of the remaining sugar *O*-trimethylsilyl groups by refluxing in methanol, the primary amino group was *N*-acylated by adding an excess of ethyl trifluoroacetate to the cooled solution. Compound **3** was isolated in 42 % overall yield *via* column chromatography on silica gel.

The 5'-*O*-phosphorylated AMP derivative **4** was prepared by treatment of **3** with the mixed reagent[4] Et_3PO_4-$POCl_3$ at 0°C and purified by elution from a column of the polymeric adsorbent XAD-2. For coupling of **4** with riboflavin phosphate



Scheme 1

i) PTSA, $NH_2(CH_2)_6NH_2$, HMDS, 145 °C, 4-12 h; ii) MeOH, reflux, 2 h; iii) CF_3CO_2Et, r.t.,1 h; iv) Et_3PO_4, $POCl_3$, 0 °C, 1-2.5 h; v) 1,1'-carbonyldiimidazole, DMF, r.t., 3h; vi) FMN sodium salt, 18-crown-6, r.t. overnight, then 35-40 °C, 20 h; HPLC C18-RP column (H_2O-CH_3CN, 3:1); vii) NaOH (pH 10.5); HPLC C18-RP column (H_2O-CH_3CN, 92.5:7.5).

(FMN) to form the FAD diphosphate derivative, the commercial sodium salt of FMN was solubilized in DMF by the addition of 18-crown-6 and the phosphate group of **4** was activated as the imidazolide. Previously, lengthy procedures were utilized to convert FMN first to the triethylammonium and then to the trioctylammonium salt before conducting the coupling reaction in DMSO.[1,5] On HPLC purification two coupling products, i.e. the *N*-trifluoroacetyl derivative of **1** and the corresponding cyclic 2',3'-*O*-carbonate were isolated, in accordance with a published procedure.[6] This literature procedure was followed also for alkaline deprotection of both products at pH 10.5 and final purification of compound **1** by HPLC. Compounds **3**, **4** and **1** were characterized by NMR and mass spectrometry.

EXPERIMENTAL

N^6-(6-trifluoroacetamidohexyl)adenosine(3). A stirred mixture of inosine (5.36 g, 20 mmol), hexamethyldisilazane (15 ml, 72 mmol) and 1,6-hexanediamine (7.0 g, 60 mmol) was heated with *p*-toluenesulfonic acid (0.38 g, 0.17 mmol) at 145°C for 4-12 h until completion of the reaction. Methanol (200 ml) was added to the cooled rection mixture, which then was refluxed for 2 h. At this stage t.l.c. on silica gel using the solvent MeOH-25% NH_4OH (14:3) revealed complete conversion of inosine to the more polar N^6-(6-aminohexyl)adenosine. Ethyl trifluoroacetate (15 ml, 126 mmol) was added dropwise at room temperature and the reaction allowed to proceed for 1 h. The solution was concentrated and the residue was purified by column

chromatography (silica gel, EtOAc-MeOH, 9:1) to afford compound **3** as a solid. This product was crystallized by dissolution in methanol and cooling in the refrigerator, m.p. 160° (3.93 g, 42% overall yield). M.S. (EI) m/z 463 (MH^+), 445, 373, 359, 331, 204, 148. ^1H NMR (400 MHz, DMSO-d_6) δ 1.33 (m, CH_2, 8 H), 1.34 (m, CH_2), 1.345 (m, CH_2), 3.20 (q, J = 7 Hz, $CH_2NHCOCF_3$), 3.62 (m, CH_2NH), 3.73 (m, H-5'), 4.04 (q, J = 3 Hz, H-4'), 4.2 (q, J = 4 Hz, H-3'), 4.68 (q, J = 6 Hz, H-2'), 5.28 (d, J = 4 Hz, OH-3'), 5.53 (d, J = 6 Hz, H-1'), 5.57 (dd, J = 7 and 4 Hz, OH-5'), 5.97 (d, J = 6 Hz, OH-2'), 7.90 (s, br, NH), 8.28 (s, br, H-2 or H-8), 8.38 (s, H-8 or H-2), 9.40 (t, J = 5 Hz, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.1, 28.3, 29.1, 61.8 (CH_2), 70.9 (CH), 73.7 (CH), 86.1(CH), 88.2 (CH), 116.1 (q, J = 290 Hz, CF_3), 119.9 (C-5), 139.8 (C-8), 148.2 (C-4), 152.5 (C-2), 154.8 (C-6), 157.25 (qq, J = 35 and 4 Hz, $COCF_3$).

N^6-(6-Trifluoroacetamidohexyl)AMP(4). Compound **2** (2.90 g, 6.26 mmol) was dissolved in Et_3PO_4 (10 ml) and to the cooled (0°C) solution was added $POCl_3$ (1.10 ml, 11.8 mmol). After 75 min ice-water (5 ml) was added, and the mixture was stirred for 15 min. The aqueous solution was adjusted to pH 7 by portionwise addition (under cooling) of 0.5 M NH_4OAc (*ca.* 80 ml). At this stage t.l.c. (silica, MeOH-25% NH_4OH, 14:1) revealed compound **4** (R_f = 0.4) as the major product and starting compound **3** (R_f = 0.6) as a trace component. The solution was evaporated to dryness, the resulting semisolid was triturated 4 times with boiling ether and the solid product collected by decantation. This product was dissolved in a minimal amount of hot water (*ca.* 150 ml) and the cooled solution was applied to a column of XAD-2 (65 cm x 2.5 cm). The column was eluted at a rate of 1-2 ml/min, first with water (800 ml) and then with MeOH-water (1:1) (800 ml). Fractions containing the product were collected (after detection by spraying t.l.c. plates with p-anisaldehyde in EtOH and heating at 100 °C) and evaporated to afford compound **4** (1.90 g, 56%) as a solid, m.p. 166-168°. M.S. (FAB, glycerol), m/z 543 (MH^+), 565 (M+Na), 581 (M+K), 635 (MH+glycerol), 331 (BH^+). ^1H NMR (400 MHz, D_2O) δ 1.38 (m, CH_2, 8 H), 1.55 (m, CH_2), 1.67 (m, CH_2), 3.25 (t, J = 7 Hz, $CH_2NHCOCF_3$), 3.52 (m, CH_2NH), 4.12 (m, H-5'), 4.38 (m, H-4'), 4.48 (t, J = 4 Hz, H-3'), 4.72 (t, J = 6 Hz, H-2'), 6.10 (d, J= 6 Hz, H-1') (assignments confirmed by selective decoupling of H-1', H-2', H-3', H-4', and H-5'), 8.18 (s, br, H-2 or H-8), 8.42 (s, H-8 or H-2). ^{13}C NMR (63 MHz, D_2O) δ 28.5, 30.5, 31.2, 42.6, 43.9, 87.3 (CH_2OP), 73.3 (CH), 77.3 (CH), 88.9 ($CHCH_2OP$), 90.0 (CH).

N^6(6-Aminohexyl)FAD (1). Compound **4** (600 mg, 1.11 mmol) was dried by evaporation of a DMF solution on a rotary evaporator attached to a vacuum pump, redissolved in DMF (10 ml), and reacted with carbonyldiimidazole (1.50 g, 9.25 mmol) at r.t. for 3 h. The excess of reagent was destroyed by addition of methanol (5 ml) and the solution was evaporated at 40°C *in vacuo*. The residue was dissolved in DMF (10 ml) and the solution combined with a mixture (dried by prior evaporation with DMF) of commercial sodium riboflavin phosphate (2.0 g, 4.18 mmol) and 18-crown-6 (1.0g, 3.8 mmol) in DMF (90 ml). After being stirred at room temperature overnight, the reaction mixture was heated at 35-40°C for 20 h. At this stage t.l.c. (silica, EtOH-1M $Et_3NH_2CO_3$, 9:1) revealed almost complete conversion of the imidazolide of **4** to a mixture of the *N*-trifluoroacetyl derivatives of **1** (R_f = 0.45) and its cyclic 2',3'-*O*-carbonate (R_f = 0.6). The solution was centrifuged, decanted, and

evaporated *in vacuo*. The residue was purified by using a Waters C-18 reversed-phase Deltapak HPLC column (particle size 15 μm, length 30 cm, ID 1.9 cm). This was eluted first with potassium phosphate buffer (0.02 M, pH = 5.5)-MeCN (9:1), then with water-MeCN (3:1) to afford FMN, the *N*-trifluoroacetyl derivative of **1**, and the cyclic carbonate, respectively. The latter two fractions were combined and evaporated *in vacuo*. M.S. (FAB, glycerol) for the *N*-trifluoroacetyl derivative of **1** isolated by t.l.c., m/z 1025 (M-H+2Na), 1047 (M-2H+3Na).

For deprotection to compound **1**, the previous residue was dissolved in water (13 ml), and the pH of the aqueous solution was adjusted to 10.5 by periodical addition of 0.1 N NaOH during 1 day. The solution was neutralized to pH 6 with 0.1 N HCl, and concentrated to *ca.* 10 ml. Compound **1** was purified by using the above-mentioned C-18 reversed-phase HPLC column, which was washed with water and then eluted with water-MeCN (92.5:7.5). Fractions containing the product were evaporated to afford compound **1** as a yellow-red powder (140 mg, 14% overall from compound **4**, yield not optimized). M.S. (FAB, thioglycerol), m/z 885 (MH^+), 907 (M+Na), 923 (M+K). ^1H NMR (400 MHz, D_2O), δ 1.30 (m, CH_2, 8 H), 1.40 (m,CH_2), 1.60 (m, CH_2), 2.38 (s, CH_3), 2.42 (s, CH_3) 2.95 (m, 4 H), 4.30 (m, 4 H), 4.50 (m, 3 H), 4.90 (m), 5.90 (d, J = 6 Hz, H-1'), 7.42 (s, 1 H), 7.72 (s, 1 H), 7.81 (s, 1H), 8.37 (s, 1 H).

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