Pergamon

Tetrahedron Letters, Vol. 36, No. 12, pp. 2005-2008, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(95)00213-8

Isolation and Structure of Flutimide, a Novel Endonuclease Inhibitor of Influenza Virus

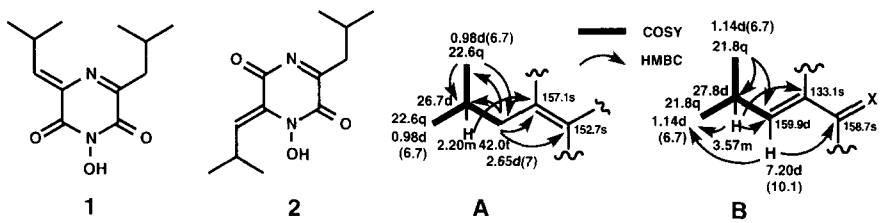
Otto D. Hensens*, Michael A. Goetz, Jerrold M. Liesch, Deborah L. Zink, Susan L. Raghoobar, Gregory L. Helms, and Sheo B. Singh

Merck Research Laboratories, P. O. Box 2000, Rahway, NJ 07065-0900 (USA)

Keywords: Flutranscription endonuclease inhibitor, 2D NMR, HMBC, SIMBA, EI-MS, FAB-MS, CI-MS

Abstract: *The isolation and structure elucidation of flutimide (**1**), an inhibitor of flutranscription endonuclease from Delitschia confertaspora, a new species, is reported. The novel natural product is characterized by N-hydroxyimide and exocyclic enamine functionalities.*

Influenza, an acute contagious respiratory disease is caused by influenza viruses A and B. The distinguishing characteristics of influenza are the mortality that can result from pulmonary infection which periodically causes epidemics. Influenza virus is a negative strand RNA virus with a segmented genome. Its mRNA synthesis is catalyzed by a virally-encoded transcription complex. This virus uniquely utilizes capped and methylated (cap 1) primers for its mRNA synthesis. Endonuclease, a unique enzyme that cleaves the capped cellular transcripts for further RNA elongation is critical for viral replication.[1] Thus inhibition of this enzyme could be potentially useful as a therapeutic target towards development of an anti-influenza drug.[2]

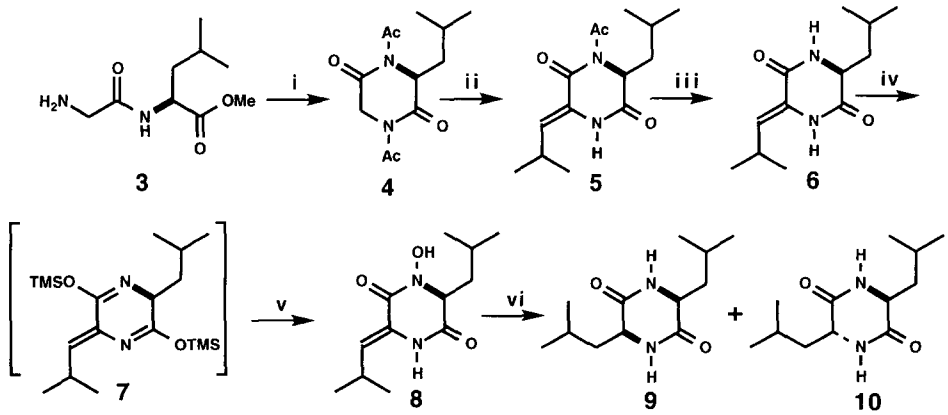


The novel natural product flutimide (**1**) was isolated from *Delitschia confertaspora*, a new species,[3] collected from dung of a dassie in Namibia, using a Flu transcription biological screen.[4] It selectively inhibits endonuclease and shows antiviral activity in cell culture. The titer of flutimide in the fermentation broth was very low and very difficult to improve.[4] Only ~1 mg of material was initially available for structure determination which did not allow a clear distinction between **1** and the isomeric hydroxamic acid structure **2** to be made based on NMR and MS evidence alone. This was resolved in favor of **1** by synthetic and degradative studies when more of **1** became available. We report here on the isolation and structural studies of flutimide, corroboration of which was obtained by an unambiguous synthesis from L-leucine.[5]

Extraction of flutimide from fermentations was accomplished by treating broths with either ethyl acetate or methyl ethyl ketone, on the condition of operating at pH 2-2.5 (acidification with conc. H_3PO_4) rather than at the original pH (usually 6.5). Concentration of the extract was followed by a gel filtration step on Sephadex LH-20 in methanol and silica gel column chromatography on EM silica (methylene chloride to methanol gradient). Final purification was achieved by HPLC on a Whatman Partisil-5 ODS-3 column operated at room temperature and eluted with a gradient of acetonitrile in 0.1% TFA. In larger scale preparations, difficulties encountered in dealing with whole-broth extracts were avoided by simply first filtering the fermentation; the de-watered mycelial cake was stirred briefly with ethyl acetate to remove large amounts of non-polar impurities, then extracted with a 1:1 mixture of MEK and 0.01M H_3PO_4. The organic phase was pooled with the broth filtrate extract prior to the gel filtration step.

^{13}C NMR data of flutimide [FTIR (ZnSe): 3241 (br), 1674, 1626, 1467, 1237, 937 cm-1; UV λ_{max} (MeOH) : 270 , 360 (sh) nm] in CD_2Cl_2 at 125 MHz, indicated 12 carbons and 17 carbon-bound protons in agreement with the HR-EIMS derived empirical formula $C_{12}H_{18}N_2O_3$ (found m/z 238.1316, calculated 238.1317) assuming one active proton. This was supported by the formation of a mono-TMS derivative. COSY and ^1H NMR decoupling studies at 500 MHz indicated the presence of isobutyl and isobutylidene groups which could be elaborated to partial fragments **A** and **B** using the ^1H detected long-range ^1H-^{13}C HMBC experiment optimized for 10 Hz. By EI-MS, fragment ions were observed at m/z 221.1290 ($C_{12}H_{17}N_2O_2$, calc 221.1290) and m/z 195.0770 ($C_9H_{11}N_2O_3$, calc 195.0770), corresponding to losses of OH and the sidechain (C_3H_7) respectively. The $[M\text{-}OH]^+$ loss was confirmed by spiking the sample with CD_3OD whereupon the $[M\text{-}OD]^+$ fragment was observed from the deuterated molecular ion at m/z 239. This is consistent with the presence of an N-OH functionality in flutimide which was confirmed by a ^2H-isotope ^{13}C induced shift experiment by comparing spectra in CD_3OD and CD_3OH. No difference in ^{13}C shifts were observed larger than 0.01 ppm ruling out an OH or NH group bound to carbon.

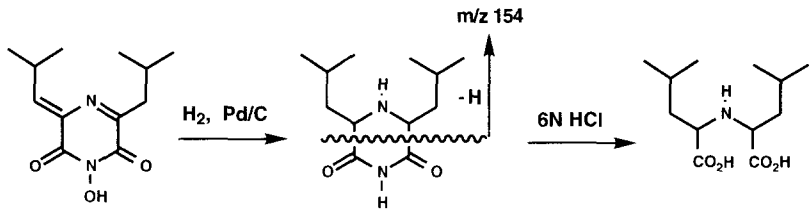
Scheme 1



i, $NaHCO_3$, EtOH, reflux, then Ac_2O, 130^{0}C; ii, *t*-BuOK, DMF-*t*-BuOH, Me_2CHCHO; iii, $NH_2NH_2.H_2O$; iv, HMDS, trace of TMSCl, reflux; v, MoO_5.Py.HMPA; vi, H_2, Pd/C

Based on these data the N-hydroxyimide (**1**) and hydroxamic acid structures (**2**) were advanced. The *Z* configuration of the exocyclic double bond followed from a 1D HMBC (SIMBA)[6] experiment. Selective irradiation of the carbon at 158.7 ppm gave a doublet of doublets for the ^{1}H resonance at δ7.20 [$^3J_{HH}$ = 10.1 Hz; $^3J_{CH}$ = 3.3 Hz] where the small $^3J_{CH}$ is consistent with a *syn* relationship between the vinylic proton and the carbonyl group.[7] We initially favored **2** based on ^{1}H chemical shift arguments and on our inability to prepare the TMS derivative of the original small sample. Synthetic studies were therefore initiated as outlined in Scheme 1 which yielded the *E* and *Z* isomeric structures of **8** as intermediates (only the major *E* isomer is shown for structures **5-8**).[8] These were expected to produce the same hydrogenation product as would have been expected for **2**. Actual hydrogenation of **8** with Pd/C was accompanied with reductive cleavage of the N-OH group to give a 95:5 mixture of **9** and **10**[11,12], different from the symmetrical, *des* N-hydroxy product **11**[13] obtained under identical conditions from **1** (Scheme 2). The CI mass spectrum of compound **11** gave a parent ion at m/z

Scheme 2



1 **11** **12**

227 (M+H). A facile elimination of CO was observed by both CIMS and EIMS to give an ion at m/z 199 (M-CO+H) and 197 (M-CO-H) respectively. This type of facile loss of CO was not observed in spectra of **9** and **10**. The most critical EI-MS fragment of **11** was observed at m/z 154.1603 [$C_4H_9CH{=}N{=}CHC_4H_9$]$^+$ as shown, which clearly supports the imide structure. More significantly, acid hydrolysis of **11** yielded the dicarboxylic acid **12** which was characterized by GC-MS analysis as a TMS derivative. Proto- and perdeutero-TMS derivatives were prepared separately to allow determination of the number of TMS groups present in observed ions. The di-TMS derivative of **12** was observed and it exhibited typical TMS-amino acid fragment ions[14] at m/z 374 (di-TMS M$^+$ - Me), 346 (374 - CO), and 272 (di-TMS M$^+$ - $CO_2Si(CH_3)_3$). On the other hand **9** and **10** gave only leucine as expected (observed m/z 275 (di-TMS M$^+$), 260 (di-TMS M$^+$ - Me), and 158 (di-TMS M$^+$ - $CO_2Si(CH_3)_3$). The structure of flutimide was therefore assigned as **1**.

Flutimide is the first natural product shown to inhibit the cap-dependent endonuclease of influenza virus. Previously,[2] a series of 4-substituted 2,4-dioxobutanoic acids were found to inhibit the endonuclease exhibiting IC_{50}s in the range 0.2 to 29.0 uM compared to a value of ~3μM for flutimide.[4]

References and Notes

1. Plotch, S. J.; Bouloy, M.; Ulmanen, I.; Krug, R. M. *Cell* **1981**, *23*, 847-858.
2. Tomassini J.; Davies, M. E.; Armstron, M. E.; Hastings, J.; Hazuda, D.; Lewis, J.; McClements, W.; Wolfe, A.; Selnick, H.; Baldwin, J.; Bourgeois, M.; Ponticello, G.; Raszilowski, E.; Smith, G.; Tebben, A. *Antimicrob. Agents Chemother.* **1994**,*38*, 2827.
3. Pelaez, F.; Polishook, J. D.; Valldosera, M.; Guarro, J. *Mycotaxon* **1994**, *50*, 115-122.

4. Tomassini J.; Lingham, R.B.; et al. submitted to *Antimicrob. Agents Chemother.*
5. Singh, S. B. accompanying paper.
6. Crouch, R.C.; Martin, G. E. *J. Magn. Reson.* **1991**, *92*, 189-194.
7. Vogeli, U.; Von Philipsborn, W. *Org. Magn. Reson.* **1975**, *7*, 617-627.
8. Scheme 1: Fischer esterification of Gly-L-Leu gave **3** which on refluxing in $NaHCO_3$/EtOH and acetylation gave **4**. Aldol condensation with isobutyraldehyde gave a 7:3 mixture of *Z* and *E* isomers **5** (CI-MS m/z 267 $[M+H]^+$) in >70% yield which were differentiated by NOE measurements. The marked difference in olefinic and isopropyl CH chemical shifts in $CDCl_3$ [*E*, δ5.58 (d, *J* = 9.9 Hz)/3.53m; *Z*, δ6.15 (d, *J* =10.2 Hz)/ 2.71m] has precedence.[9] In this step aldol condensation is followed by acyl migration and base-mediated acetate elimination. After deacetylation to **6** (*E/Z*), silylation under reflux to **7** followed by careful removal of reagent and *in situ* oxidation with MoO_5.pyridine.HMPA (prepared fresh in three steps)[10] gave **8** (*E/Z*) as well as aromatized product by elimination of water.
9. Robins, D. J.; Sefton, M. A. *Phytochemistry* **1984**, *23*, 200-201.
10. Rigby, J. H.; Qabar, M. *J. Org. Chem.* **1989**, *54*, 5852-5853.
11. **9**: Colorless oil: CIMS: m/z 227 $[M+H]^+$; ^1H NMR (CD_3OD, 400 MHz): δ0.95 (3H, d, *J* = 6.8 Hz), 0.97 (3H, d, *J* = 6.8 Hz), 1.61 (1H, ddd, *J* = 5.6, 8.8, 14.0 Hz), 1.72 (1H, ddd, *J* = 4.8, 8.4, 13.6 Hz), 1.84 (1H, m), 3.90 (1H, dd, *J* = 4.8, 8.8 Hz); ^{13}C NMR (CD_3OD, 100 MHz): 22.1 (2 x Me), 23.6 (2 x Me), 25.5 (2 x CH), 45.9 (2 x CH_2), 54.9 (2 x CH), 171.3 (2 x CON) ppm. The major product **9** is symmetrical judging from its ^1H and ^{13}C NMR spectra. It was therefore assigned the *S,S* diastereomer consistent with previous work on the asymmetric reduction of α,β-dehydroamino acid residues in cyclodipeptides.[12]
12. Kanmera, T.; Lee, S.; Aoyagi, H.; Izumiya, N. *Int. J. Peptide Protein Res.* **1980**, *16*, 280-290.
13. **11**: Colorless oil; FTIR (ZnSe): 3299 (br), 1743 (sh), 1690, 1467, 1204 cm-1; ^1H NMR (CD_3OD, 400 MHz): δ0.94 (2 x 3H, d, *J* = 6.4 Hz), 0.97 (2 x 3H, d, *J* = 6.4 Hz), 1.87-1.98 (2 x 3H, m), 3.61 (2 x 1H, dd, *J* = 4.0, 9.6 Hz); ^{13}C NMR (CD_3OD, 100 MHz): 21.9 (2 x Me), 23.6 (2 x Me), 26.0 (2 x CH), 41.0 (2 x CH2), 58.8 (2 x CH), 173.6 (2 x CON) ppm.
14. Leimer, K.; Rice, R.; Gehrke, C. *J. Chromatogr.* **1977**, *141*, 355-375.

(*Received in USA* 7 *December* 1994; *revised* 24 *January* 1995; *accepted* 26 *January* 1995)