3-ANILINO-L-ALANINE, STRUCTURAL DETERMINATION OF UV-5, A CONTAMINANT IN EMS-ASSOCIATED L-TRYPTOPHAN SAMPLES

Yukihiro GODA, Junko SUZUKI, Tamio MAITANI, Kunitoshi YOSHIHIRA, Mitsuharu TAKEDA and Mitsuru UCHIYAMA*

National Institute of Hygienic Sciences (NIHS), 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

An aniline derivative which corresponds to UV-5 in the preceding paper[1] was isolated from the case L-tryptophan sample associated with eosinophilia-myalgia syndrome (EMS). By spectroscopic analyses, the structure was identified as 3-anilinoalanine. The compound was optically active, and the stereochemistry of alanine moiety was determined as L, by comparing the specific rotation with a synthesized 3-anilino-L-alanine.

KEYWORDS 3-anilino-L-alanine; tryptophan; eosinophilia-myalgia syndrome (EMS); aniline derivative

The eosinophilia-myalgia syndrome (EMS) associated with the consumption of L-tryptophan products has been recognized worldwide. According to the report,[2] most of the patients had consumed the tryptophan products that were derived from bulk L-tryptophan powder (the case tryptophan) manufactured by a single company between October 1988 and June 1989. Although a considerable effort has focused on characterization and identification of a compound (or compounds) which causes EMS, clear results have not been reported yet, since a bio-assay system reproducing EMS by the case tryptophan has not been developed. In the preceding paper[1] we described the characterization of contaminants in the case tryptophan by high-performance liquid chromatography (HPLC) and reported the two contaminants, designated as UV-5 and UV-15, which were significantly associated with the case tryptophan. The structure of UV-15, corresponding to peak E[2] or contaminant 97,[3] was elucidated as a di-L-tryptophan aminal of acetaldehyde by several groups[4] and finally confirmed as 1,1'-ethylidenebis(L-tryptophan) by Smith *et al.*[3] For UV-5, we suggested[5] the structure as an aniline derivative based on by some spectroscopic data; however, we could not confirm it definitely because of the small quantity available. In this paper, we describe the isolation of UV-5 by large-scale HPLC and its structural determination mainly by spectroscopic analyses.

Before a large-scale separation, primary HPLC analyses were performed on the respective manufacturing lots of the case tryptophan, and the L-tryptophan of lot No.67116202 (No.24 in the preceding paper[1]) was selected because of the small amounts of the compounds interfering with the peak of UV-5. Then the L-tryptophan was recrystallized to concentrate UV-5 into the mother liquor, which was subjected to isocratic preparative HPLC[6] for large-scale separation. After repeated chromatographies, 2 mg of UV-5 was obtained from about 150 g of the case lot.

An HPLC-MS[7] analysis of UV-5 showed quasi-molecular ion peak at *m/z* 181. The high-resolution MS analysis of the peak gave the molecular formula as $C_9H_{13}O_2N_2$. The IR spectrum[8] showed

Table I. ^{1}H-NMR Chemical Shifts of UV-5 (400 MHz, δ Value) at 25°C

Proton	Solvent		
	d_1-TFA/D_2O (1/99)	DCl/D_2O (3/997)	d_1-TFA/d_6-DMSO (5/995)
CH	4.19(dd, J=7.0, 5.9 Hz)	4.09(t, J=6.4 Hz)	4.06(brs)
CH_2-Ha	3.69(dd, J=5.9, 14.2 Hz)	3.61(dd, J=6.4, 14.4 Hz)	3.49(d, J=5.8 Hz)
CH_2-Hb	3.83(dd, J=7.0, 14.2 Hz)	3.72(dd, J=6.4, 14.4 Hz)	3.49(d, J=5.8 Hz)
NH_2	Not detected	Not detected	8.23(brs)
Aromatic C2,6-H_2	7.11(d, J=8.7 Hz)	6.95(d, J=9.5 Hz)	6.59(d, J=7.6 Hz)
Aromatic C3,5-H_2	7.35(m)	7.27(t, J=9.5 Hz)	7.12(t, J=7.6 Hz)
Aromatic C4-H	7.15(t, J=6.8 Hz)	6.98(t, J=9.5 Hz)	6.58(t, J=7.6 Hz)

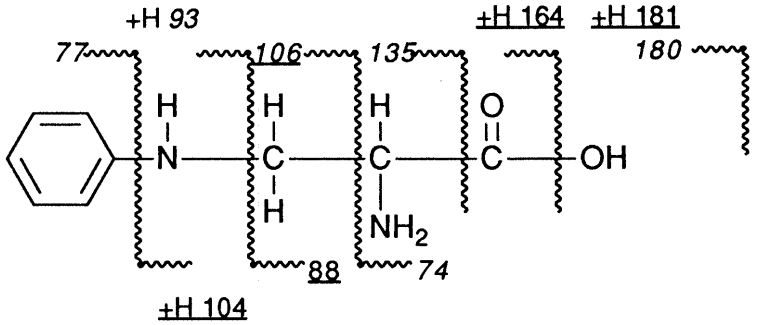

Fig. 1. Fragmentation of UV-5
italic: detected by EI-MS; under line: detected by ESI-MS/MS or HPLC-MS.

absorption bands at 3470 cm^{-1} and 3300 cm^{-1}, which indicated the presence of amino and imino groups, respectively. The ^{1}H-NMR spectrum measured in 1% d_1-TFA in D_2O and 0.3% DCl in D_2O showed signals caused by five aromatic protons of an A_2BX_2-type and three aliphatic protons of an ABX-type (Table I). The ^{13}C-NMR studies[9] suggested the presence of six aromatic carbons, two aliphatic carbons and a carboxyl carbon. These data and the fragmentation in several kinds of MS analyses (Fig.1) suggested that UV-5 was 3-anilinoalanine. In addition, heteronuclear multiple-bond connectivity (HMBC) spectrum indicated the correlation between the aromatic quarternary carbon and the methylene protons which supported the structure. UV-5 was optically active and the $[\alpha]_D$ value at 25°C was +2.5° in H_2O-CH_3CN-TFA (90:10:0.1)

In order to determine the absolute configuration, 3-anilino-L-alanine was synthesized from aniline and 3-chloro-L-alanine. After repeated HPLC purifications, the synthetic compound showed the same spectral data (including specific rotation) as those of UV-5 from the case tryptophan. Consequently, the structure of UV-5 was determined to be 3-anilino-L-alanine. This compound has not been reported so far. Quantitative HPLC analyses[10] revealed that case tryptophan contained 3-anilino-L-alanine at a level of 0.01 %.

In 1% d_1-TFA in D_2O and 0.3 % DCl in D_2O, non-equivalent methylene protons were detected by ^{1}H-NMR (Table I). The fact is most likely due to the hydrogen bonding between imino and carboxyl groups since the formation of the hydrogen bonding strongly depresses the free rotaion of alanine moiety. A higher-field shifted signal of the carboxyl carbon at δ169.4[9] also supports the existence of the hydrogen bonding. On the other hand, it seems that the hydrogen bonding does not exist in d_6-DMSO containing

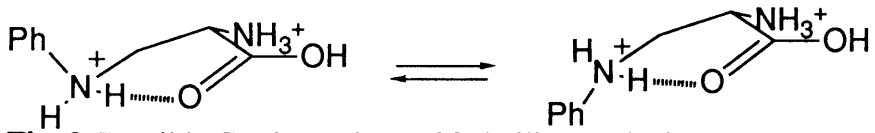

Fig. 2. Possible Conformations of 3-Anilino-L-alanine

0.5% d_1-TFA since the methylene proton signal was observed as a doublet.

When the hydrogen bonding is formed, two conformations are possible, as shown in Fig. 2. In fact, two distinguishable ABX type signals which might correspond to the two conformations were observed in the aliphatic field of ^{1}H-NMR spectrum in the solvent of 0.1% d_1-TFA in D_2O at 25°C. On the other hand, under more acidic conditions such as 1% d_1-TFA in D_2O and 0.3% DCl in D_2O, it seems that the equilibrium between the two conformations is inclined to one of the conformations, since a characteristic ABX type coupling was observed in the aliphatic field of the spectrum.

Recently Kilbourne *et al.* reviewed the toxic oil syndrome that occurred epidemically in Spain in 1981.[11] In the review they mentioned that the EMS associated with the case tryptophan resembles the toxic oil syndrome and that the toxic oil syndrome has been linked to the ingestion of oil mixtures containing rapeseed oil denatured with aniline. Furthermore, they speculated that the causal agent might be a reaction product of aniline with some oil component, although aniline itself did not cause the syndrome. Therefore, the toxicological implication is that 3-anilino-L-alanine is a suspected causal substance of EMS. Toxicological experiments are currently under way.

ACKNOWLEDGMENTS The authors thank Showa Denko K.K. for providing the case tryptophan and for cooperation in preparative HPLC. The authors also thank Dr. Y. Ohashi (the University of Tokyo), Dr. H. Noguchi (the University of Tokyo) and K. Kanohta (NIHS) for measurements of ESI-MS/MS, HMBC by 500 MHz NMR and HPLC-MS, respectively.

REFERENCES AND NOTES

1) T. Toyo'oka, T. Yamazaki, T. Tanimoto, K. Sato, M. Sato, M. Toyoda, M. Ishibashi, K. Yoshihira and M. Uchiyama, *Chem. Pharm. Bull.*, **39**, 820 (1991).
2) E.A. Belongia, C.W. Hedberg, G.J. Gleich, K.E. White, A.N. Mayeno, D.A. Loegering, S.L. Dunnette, P.L. Pirie, K.L. MacDonald and M.T. Osterholm, *N. Engl. J. Med.*, **323**, 357(1990).
3) M.J. Smith, E.P. Mazzola, T.J. Farrell, J.A. Sphon, S.W. Page, D. Ashley, S.R. Sirimanne, R.H. Hill, Jr. and L.L. Needham, *Tetrahedron Lett.*, **32**, 991 (1991).
4) Update: Analysis of L-tryptophan for etiology of eosinophilia-myalgia syndrome, *MMWR*, **39**, 789 (1990); K. Sakimoto, *N. Engl. J. Med.*, **323**, 992(1990).
5) Y. Goda, J. Suzuki, K. Sato, K. Yoshihira and M. Uchiyama, Abstracts of Papers, 61st Annual Meeting of the Food Hygienic Society of Japan, Tokyo, May 1991, p62.
6) "HPLC condition" column: Inertosil Prep-ODS (30 x250 mm), Cosmosil 15C18-AR (20 x250 mm) x2 or Shodex ODS C18 (100x355 mm); flow rate: 20-35 ml/min (except for Shodex) or 150 ml/min (Shodex); temperature: 40°C (except for Shodex) or ambient (Shodex); detection: 280 nm; solvents: H_2O-CH_3CN-TFA (90:10:0.1) except for final purification or H_2O-CH_3CN (92:8) for final purification.
7) HPLC-MS was performed with VG-ZAB-SEQ equipped with a JEOL 880-PU pump.
8) IR (KBr) cm^{-1}: 3470, 3300, 1615, 1550, 1460, 1395, 1340, 1135, 1090.
9) Measured in 0.3% DCl in D_2O, δ: 169.4(CO), 142.3(Ar-1), 128.1(Ar-3 or Ar-5), 128.0(Ar-3 or Ar-5), 119.5(Ar-4), 113.8(Ar-2 and Ar-6), 50.0(CH-NH_2), 43.1(CH_2).
10) HPLC was performed according to the condition described in the preceding paper.[1]
11) E.M. Kilbourne, M.P. de la Paz, I.A. Borda, M.D. Ruis-Navarro, R.M. Philen and H. Falk, *J. Am. Coll.Cardio.*, **18**, 711 (1991).

(Received June 5, 1992)