AN OLIGOMERIC HYDROXYPHENYLALANINE IN MALIGNANT MELANOMA: A NEW TYPE OF MELANOGEN

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Received October 4, 1974

SUMMARY. A new compound designated MM2 has been isolated by ion exchange chromatography from tumor extracts and urine of patients with malignant melanoma. This material was purified by G-25 sephadex chromatography and studied by standard spectroscopic techniques including nuclear magnetic resonance, ultraviolet, infrared, and mass spectrometry. These data are consistent with an oligomeric structure of four dihydroxyphenylalanine subunits with subunit bonding made between phenyl rings. The appearance of large quantities of such an oligomer in malignant melanoma suggests the existance of intermediate stages of polymerization of melanin precursors before the final melanin high polymer is formed.

INTRODUCTION

The melanogens are frequently observed in the urine of patients with malignant melanoma and are hypothesized to be either precursors of melanin or metabolic byproducts of the melanin biosynthetic pathway (1,2). Duchoñ, et. al. have separated the melanogens into two groups, the phenols and indoles, which are thought to be monomeric dihydroxyphenols or indoles (1,2). Duchon and Humbel suggest that these compounds are derived from 3,4-dihdroxyphenylalanine or 5,6-dihydroxyindole-2-carboxylic acid by decarboxylation, methylation, sulfonation, or glucurination reactions (1,2,3). We report here the partial elucidation of the structure of a new compound, MM2*, obtained from tumor extracts and urine of patients with malignant melanoma. From our data MM2 appears to be an oligomeric dihydroxyphenylalanine.

MATERIALS AND METHODS

Urine from patients was concentrated 20 fold by evaporation (37°C).

*MM = Malignant Melanoma

The pH was adjusted to 2.2 with sulfosalicylic acid to precipitate protein. The urine concentrate was applied to a Spinco type 150 A sulfonated styrene-8% divinylbenzene resin column on a Beckman-Spinco 120 amino acid analyzer. The column was then washed with 0.2N citrate buffer, pH 3.25 at 30°C, and then 3 compounds, designated MM1, MM2 and MM3, were eluted with 0.2N citrate, pH 4.25 at 50°C.

Tumor tissue extracts were prepared by homogenizing 20 g of frozen tissue in 200 ml of 1% aqueous picric acid. The homogenate was centrifuged and the supernatant fluid was passed through a Dowex 2x8 resin, chloride form, to remove the picric acid.

Depending upon the patient and the stage of disease, MM2 was obtained in quantities ranging from 0.1 to 1.0 mg per ml of urine and up to 1.0 mg per gm of tumor tissue.

Ninhydrin positive compounds were detected spectrophotometrically. One compound, MM1, elutes between leucine and tyrosine. Two compounds, MM2 and MM3, were observed to elute after phenylalanine.** (Figure 1) Rechromatography of each material on Whatman #3 paper in butanol acetic acid-H_2O (12:3:5) was performed to determine purity. One ninhydrin positive

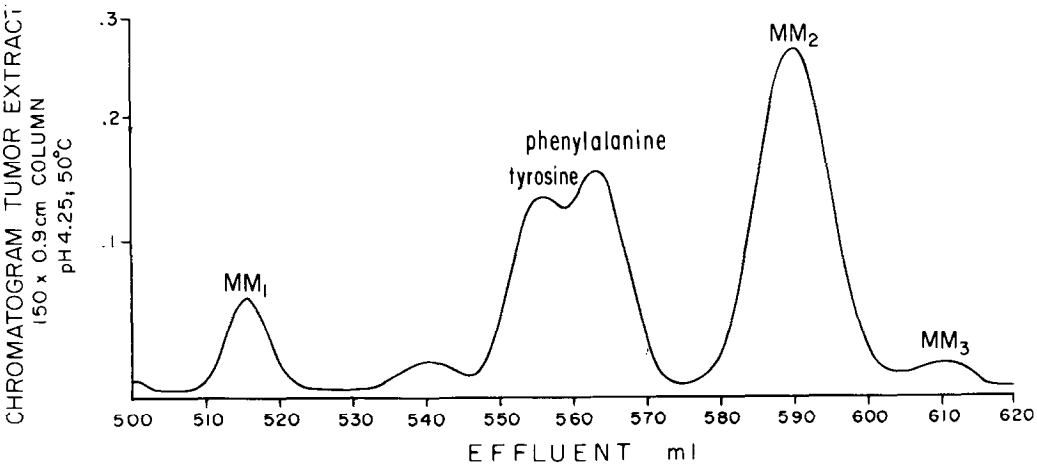


FIGURE 1. Amino acid analyzer chromatogram tumor extract. 150 x 0.9 cm column, 0.2 N citrate buffer, pH 4.25; 50°C.

** On a Durrum DC-1 column these 3 compounds coelute with phenylalanine.

spot was shown for each material at Rf values of 0.16 (MM1), 0.14 (MM2) and 0.21 (MM3).

The MM2 fraction from the Spinco column was chromatographed five times on G-25 Sephadex in 0.1 M acetic acid to remove citrate which elutes before MM2. Lyophilizing the column fractions yielded an amorphous, nonhydroscopic white solid which was stable in room air. The compound MM2 thus purified chromatographs as a single species on two dimensional chromatography in the solvent pairs butanol : acetic acid : water (first) and phenol : water (second).

MM2 is unstable at pH 7.5 at 25°C, yielding several ninhydrin positive and negative peaks on paper chromatography. However, 70% of MM2 is recovered unchanged after heating at 110°C for 24 hr in 6N HCl.

Infrared (IR) spectra were recorded on a Perkin-Elmer 137 using solid KBr. Ultraviolet (UV) spectra were recorded on a Unicam SP 800 at 25°C. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA 100 at 25°C. Mass spectra of MM2 were run on an AEI, MS9 double focusing mass spectrometer at 150°C.

Elemental analyses were carried out by Clark Microanalytical Laboratory, Urbana, Illinois.

RESULTS

The IR spectrum of MM2 in compressed KBr showed strong peaks at 2.9μ, 3.2μ 3.4μ, 6.12μ, 6.30μ, 6.80μ, and 7.15μ. The peak at 6.2μ is consistent with a carboxylic acid carbonyl. There is a conspicuous absence of an amide carbonyl at 6.0μ.

The ultraviolet spectra of MM2 obtained from urine and from tumor tissue are shown in Figure 2. There is an absorption maximum at 287 mμ and a shoulder at 250 mμ. The concentrations shown in Figure 2 are based on leucine equivalents by the ninhydrin reaction. From these data the ε values (molar absorptivity) in Table 1 were calculated for MM2. For comparison, ε's are given for several 3,4-dihydroxybenzene compounds. The close simi-

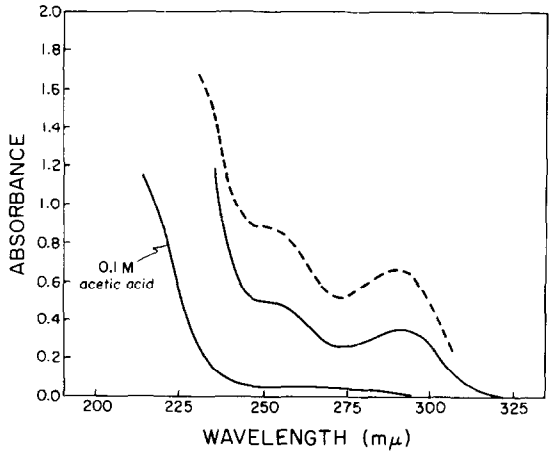


FIGURE 2. Ultraviolet spectra of purified MM2 isolated from tissue (———) and isolated from urine (-----). Concentrations are 0.09 mole per ml for tissue and 0.22 mole per ml for urine isolated MM2. Solvent is 0.1M acetic acid.

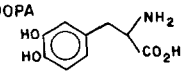
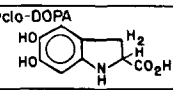
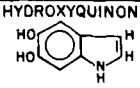
STRUCTURE	λmax, nm	ε x10³	λ(2), nm	ε x10³	Ref.
MM2 (0.1M, Acetic Acid)	287	3.0	250 sh	4.1	
DOPA 	281	2.8	220 sh	6.0	1
cyclo-DOPA 	285	4.1	228 sh	3.9	2 (20% HCl)
DIHYDROXYQUINONE 	302	6.2	275 max	4.0	3 (EtOH)

TABLE 1. UV spectral data for MM2 and dihydroxyphenylalanine derivatives. (7,8,9)

larity of the UV spectrum of MM2 to those of dihydroxyphenylalanine (DOPA) and cyclo-DOPA (an indolene) suggest that MM2 also has a dihydroxybenzene chromophore. Assuming that MM2 is a dihydroxybenzene derivative, the spectral data in Table 1 indicate there are probably one or two phenyl rings per ninhydrin positive nitrogen.

Thirty ml of 0.9μ mole/ml MM2 in 0.1 M acetic acid were lyophilized and 9.5 mg of white solid were obtained. The equivalent weight of MM2 is calculated to be 350 (± 10%) on the basis of ninhydrin positive nitrogens.

In the mass spectrum of MM2, a clean fragmentation pattern was ob-

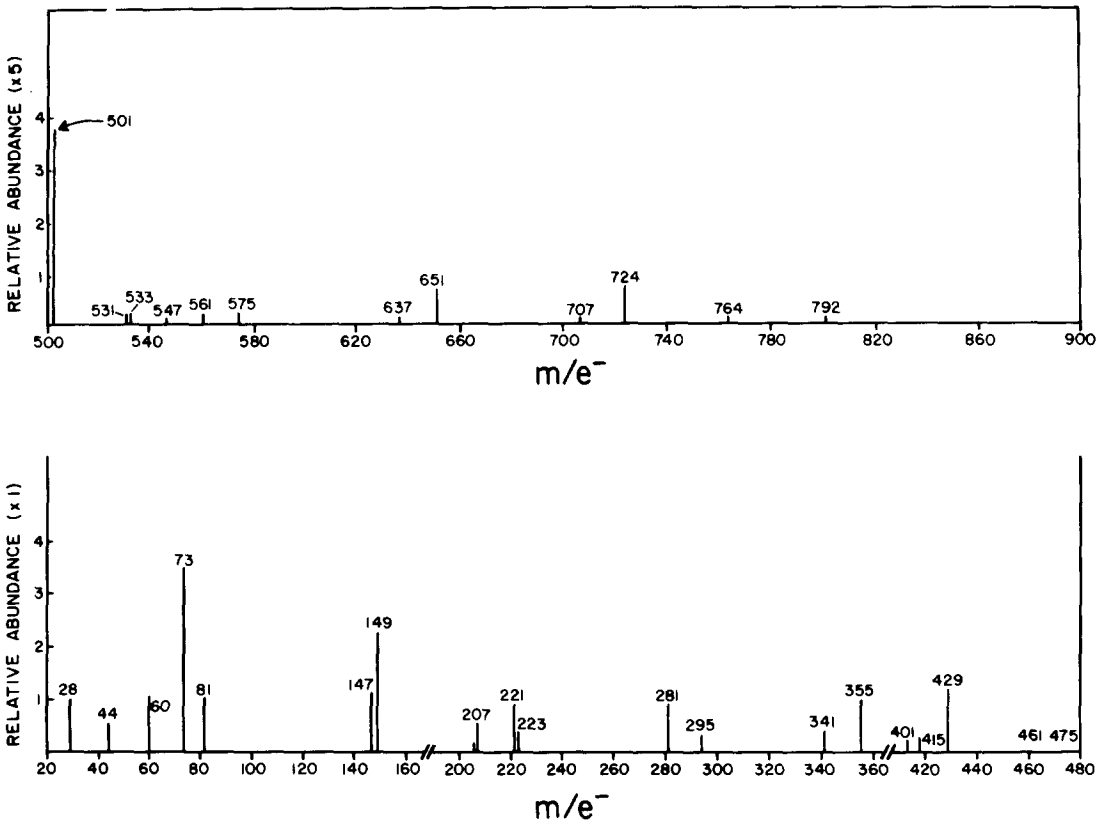


FIGURE 3. Mass spectrum of MM2.

tained (Figure 3). Strong peaks were observed at m/e = 792 ± 5, 724, 651, 501, 429 and 355. Weak peaks appeared at 858 and 836 ± 5. Recurrent in the fragmentation pattern was the loss of m/e = 28 (CO) which has been found to be characteristic for hydroquinones and phenols (4).

The NMR spectrum of MM2 in D_2O shows a doublet (2H) at δ=7.0, and multiplets at δ=3.9 (2H), 3.4 (2H) and δ=3.2 (3H). There were no resolvable aliphatic protons.

Elemental analyses for Carbon, Hydrogen, and Nitrogen showed C = 49.8%, H = 6.3% and N = 8.7%.

DISCUSSION

Considering the mass spectral data with the equivalent weight of MM2 by the ninhydrin reaction, the molecular weight of MM2 could be in the range

of 350 or 700, depending on whether there are one or two ninhydrin positive nitrogens per molecule. To be consistent with the nitrogen elemental analysis there must be two nitrogens for each 350 increment in molecular weight. The UV spectral data suggests that there is at least one aromatic chromophore per nitrogen (Table 1).

Combining these considerations with the elemental analyses and IR spectral data, tentative structures for MM2 are drawn by representing MM2 as a tetramer of dihydroxyphenylalanine with cyclization of two subunits to form indolenes. Such tentative structures would have molecular weights of about 780 and an NMR spectra closely approximating that observed if the juncture of phenylalanine subunits is achieved between phenyl rings (5).

Assuming the MM2 is on the pathway to melanin formation, the intermediacy of a dihydroxyphenylalanine oligomer would indicate an intermediate degree of structural organization before the final polymerization of melanin is achieved. Establishing the complete structure of MM2, a pure, easily characterized compound, should prove useful in the difficult task of understanding in greater detail the structure of melanin (6).

ACKNOWLEDGMENTS

We thank Bernard L. Trumpower and Gibbons Cornwell for useful advice and Jerry Dudek and William Hull for assistance in obtaining NMR and mass spectra.

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