Isolation and Identification of a Stabilized Derivative of Aldophosphamide, a Major Metabolite of Cyclophosphamide

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SUMMARY

Aldophosphamide semicarbazone was synthesized from 4-hydroxycyclophosphamide and characterized by infrared and proton magnetic resonance analysis. The synthetic specimen was shown to be identical, by thin-layer chromatographic comparison in four solvent systems, to an alkylating semicarbazone derivative of an aldehyde produced in a model oxygenase system. The structural identification of aldophosphamide semicarbazone and its identity with the product from cyclophosphamide in a model oxygenase system provides direct evidence for the intermediacy of aldophosphamide in the major metabolic pathway of cyclophosphamide.

INTRODUCTION

Cyclophosphamide (1), an important antitumor agent (9), is metabolized in mammalian liver to carboxycyclophosphamide, the major urinary metabolite (2, 13). Recently, Sladek (11) reported evidence demonstrating the generation of an alkylating aldehyde from cyclophosphamide in vivo and in vitro in biological and chemical systems. A stabilized form of the aldehyde was obtained in all of the in vitro systems in the presence of semicarbazide and was considered to be aldophosphamide semicarbazone, although isolation, purification, and structural identification were not attempted.

Because the positive identification of aldophosphamide would confirm the currently accepted, major metabolic pathway of cyclophosphamide proposed originally by Norpoth (10) and illustrated in Chart 1, the unequivocal identification of the stabilized product obtained by Sladek (11) was undertaken. Direct evidence for the generation of 4-hydroxycyclophosphamide, the initial major metabolite, has been provided recently by Connors et al. (6), who identified 4-ethoxycyclophosphamide as a product from ethanol treatment of a liver microsomal system. Phosphoramid mustard has also been identified in vivo (5, 6) and in vitro (4). Aldophosphamide, then, remained the only metabolite in the major metabolic pathway for which structural identification had not been reported.

MATERIALS AND METHODS

Diethylaldophosphamide and Diethylhomoaldophosphamide. Oxidation of diethylclophosphamide and diethyl-

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alent amount of triethyl phosphite at −20°. The solution was evaporated to dryness 5 min later to remove triethyl phosphate and any excess phosphite. The residue [4-hydroxy-cyclophosphamide, homogeneous upon TLC in acetone: chloroform (3:1), RF 0.3] in ethanol was treated with semicarbazide hydrochloride and sodium acetate. Water was added dropwise until complete solution occurred. After 15 hr at room temperature, the solution was separated by TLC in chloroform:ethanol (9:1). The alkylating band, which was detected with 4-(p-nitrobenzyl)pyridine, was eluted with methanol and filtered, and the filtrate was evaporated. The residue was analyzed by TLC in chloroform: methanol (9:1, 3:1, and 1:1) and in chloroform: ethanol (9:1) and was shown to be homogeneous. The isolated compound was subjected to mass spectral analysis by the electron impact technique and revealed only 1 chlorine-containing fragment (m/e 92, 1 Cl) and no M peak, thus yielding no useful structural information.

The IR spectrum showed broad bands at 3450 and 3210 cm⁻¹ (NH), strong bands at 1680 and 1575 cm⁻¹ (typical of semicarbazones (3)) and at 1210 cm⁻¹ (P + O), and medium intensity bands at 2965 (CH), 2920 (CH), 1430, 1130, 1085, 1040, 980, 760, and 650 cm⁻¹. PMR analysis in dimethyl sulfoxide with tetramethylsilane as internal reference gave the following results with suggested assignments: δ 2.36 to 2.56 (2H, triplet, —@@CH2O—), 3.20 to 3.46 (4H, multiplet, —CH2Cl), 3.54 to 3.80 (4H, multiplet, N—CH2—), 3.86 to 4.14 (2H, quartet, —0—Cl2—), 4.28 to 4.50 (2H, doublet, P—NH2), 6.16 [2H, singlet, —C(O)NH2I], 7.10 to 7.26 (1H, triplet, —CH=), 9.86 (1H, singlet, —N—NH—); assignments of 2.36 to 2.56, 3.86 to 4.14, and 7.10 to 7.26 were confirmed by spin decoupling; addition of D₂O resulted in the disappearance of bands 4.28 to 4.50, 6.16, and 9.86. These IR and PMR data establish the structure of aldophosphamide semicarbazone (Chart 2).

Incubation of cyclophosphamide in a model oxygenase system in the presence of semicarbazide, as described by Sladek (11), yielded an alkylating derivative of cyclophosphamide (Sladek's Metabolite H) upon subsequent TLC. The derivative was isolated by TLC in chloroform:methanol (9:1, 1:1) and in chloroform:ethanol (9:1) and was shown to be homogeneous. The isolated compound was subjected to mass spectral analysis by the electron impact technique and revealed only 1 chlorine-containing fragment (m/e 92, 1 Cl) and no M peak, thus yielding no useful structural information.

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Simultaneous TLC of synthetic aldophosphamide semicarbazone and the alkylating component obtained from the model oxygenase system in 4 solvent systems (Table 1) indicated complete identity of the synthetic specimen and the product generated from cyclophosphamide in vitro. These results substantiate the report by Sladek (11) that the alkylating aldehyde generated in the model oxygenase system is indeed aldophosphamide. Since Sladek (11) obtained the same product in the microsomal systems as in the model system, as indicated by 2-dimensional TLC, the identity of the common product is established.

It was of interest to compare TLC mobility of aldophosphamide itself with that of cyclophosphamide and 4-hydroxy-cyclophosphamide as a possible tool for facile identification. However, many attempts to prepare aldophosphamide yielded only trace amounts (12), and its extreme instability prevented direct TLC comparison. Because of this instability, the closely related analogs diethylaldophosphamide and diethylhomoaldophosphamide were synthesized. Although diethylaldophosphamide was not stable enough to allow for complete purification, TLC data were obtained at 5° and indicated its highly mobile nature (RF 0.56) in chloroform:butanol (9:1). Diethylhomoaldophosphamide does not suffer from the extreme instability.
Identification of Aldophosphamide Semicarbazone

Table 1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Model oxygenase product</th>
<th>Synthetic aldophosphamide semicarbazone</th>
<th>Cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform:methanol (9:1)</td>
<td>0.14</td>
<td>0.14</td>
<td>0.65</td>
</tr>
<tr>
<td>Chloroform:methanol (3:1)</td>
<td>0.67</td>
<td>0.67</td>
<td>0.95</td>
</tr>
<tr>
<td>Chloroform:methanol (1:1)</td>
<td>0.75</td>
<td>0.75</td>
<td>0.85</td>
</tr>
<tr>
<td>Chloroform:ethanol (9:1)</td>
<td>0.13</td>
<td>0.13</td>
<td>0.65</td>
</tr>
</tbody>
</table>


ACKNOWLEDGMENTS

The author is indebted to Dr. W. C. Coburn, Jr., and members of the Molecular Spectroscopy Section of Southern Research Institute for the spectral data presented here.

REFERENCES

1. Arnold, H., Bourdeaux, F., and Brock, N. Über Beziehungen zwischen chemischer Konstitution und cancerotoxischer Wirkung inherent in aldophosphamide and diethylaldophosphamide, although its TLC mobility would not be expected to be altered radically by the extra methylene group. Consequently, TLC mobility of this stabilized analog is probably closely indicative of the mobility of aldophosphamide. Direct TLC comparison with cyclophosphamide (Rf 0.65) and 4-hydroxycyclophosphamide (Rf 0.3) in chloroform:methanol (9:1), the 1st solvent system used by Sladek, indicated greater mobility than did TLC of the semicarbazone (Rf 0.14), whereas Sladek observed an Rf value of 0.70 for diethylmoldophosphamide. These data tend to argue against Sladek's Metabolite L (11) as aldophosphamide. Metabolite L likewise does not correspond to 4-hydroxycyclophosphamide, because TLC of 4-hydroxycyclophosphamide (Rf 0.3) in chloroform:methanol (9:1), the 1st solvent system used by Sladek, indicated greater mobility than did TLC of the semicarbazone (Rf 0.14), whereas Sladek observed an Rf value of 0.07 for diethylmoldophosphamide. Sladek's Metabolite L may possibly be a moderately stabilized derivative of aldophosphamide (e.g., an aldime derived from aldophosphamide and a protein) or of 4-hydroxycyclophosphamide; either type could conceivably react with semicarbazide to generate aldophosphamide semicarbazone. Rf values obtained in this study are consistently lower than those reported by Sladek (11) in the same solvent system, presumably because of the differences in the types of plates used and in the degree of activation of the silica gel.

In conclusion, chemical synthesis of aldophosphamide semicarbazone and comparison of the synthetic product with the semicarbazone derivative of an alkyllating aldehyde produced in a model oxygenase system have provided direct evidence for the intermediacy of aldophosphamide in the major metabolic pathway of cyclophosphamide, an important antitumor agent.
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