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Journal of Chromatography B, 767 (2002) 27–33

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Identification of the major metabolite of 2,5-bis(5-hydroxymethyl-2-thienyl)furan (NSC 652287), an antitumor agent, in the S9 subcellular fraction of dog liver cells

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Received 12 July 2001; received in revised form 24 October 2001; accepted 24 October 2001

Abstract

α-Terthienyl (**1**) is a trithiophene found widely distributed in plants. Other naturally occurring trithiophenes are less widely distributed, but nonetheless exhibit potent antiviral and cytotoxic activities. A synthetic analog of **1**, 2,5-bis(5-hydroxymethyl-2-thienyl)furan (**2**; NSC 652287) has recently been shown to possess exceptional activity and selectivity against several cell lines of the National Cancer Institute (NCI) anticancer drug screen. When incubated with the S9 subcellular fraction of dog liver cells, the concentration of **2** was observed to decline as a function of time, with a concomitant increase in a significant, time-dependent concentration of an unknown entity. The results of electron-ionization mass spectrometric analysis of the metabolite indicate an increase in 14 amu over that of **2**, leading to suspicions that either an oxidation or a methylation had occurred. Results of differential derivatization and accurate mass analysis allow us to propose that metabolism of **2** involves the biotransformation of one of the two hydroxymethyl groups of **2** into a carboxylic acid functionality. This is further supported by separate experiments involving chemical oxidation and S9 incubation of 5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxaldehyde: comparing the mass spectra and gas chromatographic retention times of the resulting products to those of the identified metabolite of **2** show all to be the same.

Keywords: 2,5-Bis(5-hydroxymethyl-2-thienyl)furan; NSC 652287

1. Introduction

α-Terthienyl (**1**, Fig. 1) was first isolated from the yellow petals of marigolds (*Tagetes erecta*) in 1947 [1]. Many trithiophenes have subsequently been shown to naturally occur in plants, although only **1** is found to be widely distributed [2]. The photoactivated antiviral and cytotoxic activities of **1** and several of its synthetic analogs have been examined [3], whereas other studies have shown certain deriva-

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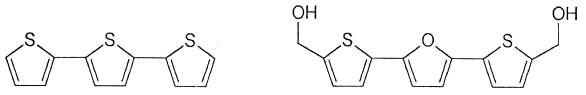
1570-0232/02/$ – see front matter
PII: S0378-4347(01)00530-8



1 2

Fig. 1. The chemical structures of α-terthienyl (**1**) and 2,5-bis(2-hydroxymethyl-5-thienyl)furan (**2**; NSC 652287).

tives of **1** to inhibit protein kinase C [4]. Recently, a synthetic analog of **1**, 2,5-bis(5-hydroxymethyl-2-thienyl)furan (**2**, Fig. 1), has shown exceptional activity and selectivity against several cell lines of the National Cancer Institute (NCI) anticancer drug screen [5]. Studies suggest that **2** promotes the formation of DNA–DNA and DNA–protein cross-links [6] and that accumulation of **2** within the cell and the cell's ability to metabolize **2** may be major factors responsible for the observed differential antiproliferative activity [7]. Thus, our efforts focused on characterizing the disposition of **2**.

2. Experimental

2.1. Chemicals

2,5-Bis(5-hydroxymethyl-2-thienyl)furan (**2**; NSC 652287) and 5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxaldehyde (NSC 672170) were obtained from the Pharmaceutical Resources Branch, DTP, DCTDC, NCI (Bethesda, MD, USA). Unless noted to the contrary, all additional solvents and reagents were obtained from commercial sources in grades appropriate for direct use.

2.2. Incubation conditions with S9 fractions

Dog liver S9 fractions (Invitro Technologies Inc., Baltimore, MD) were used to perform S9 incubations. Stock solutions of potassium phosphate buffer (1 *M*, pH 7.0), magnesium chloride (0.5 *M*), and NADPH (10 m*M*), were prepared using water, whereas a stock solution of NSC 652287 (10 m*M*) was prepared using DMSO. Freshly prepared stock solutions were maintained in an ice-water bath until use.

To 1.5-ml polypropylene microcentrifuge tubes (Eppendorf, Fremont, CA) was added 50 μl of the potassium phosphate buffer, 40 μl of the magnesium chloride solution, 200 μl of the NADPH solution, about 100 μl of S9 (the actual volume added provided a final protein concentration of 2 mg/ml in the incubation reaction mixture), and sufficient water to bring the final volume of the mixture to 990 μl. This mixture was briefly mixed by vortex action and then placed in an Eppendorf Thermomixer (set for 500 mixing strokes/min and 37°C) for 5 min. To the mixture was then added 10 μl of the NSC 652287 solution, after which mixing at 37°C continued. Two sample tubes were removed for sample processing at time intervals of 0, 5, 10, 15, 20, 45, 60, 90, 120, and 240 min.

2.3. Sample processing and chemical derivatization

The incubation mixtures from the tubes were transferred to separate conical glass centrifuge tubes. To each tube was added 5 ml of *tert*.-butyl methyl ether, whereupon each tube was tightly capped, placed on a reciprocating shaker (Eberbach, Ann Arbor, MI) for 10 min, and then centrifuged for 10 min at 2500 *g*. The organic fractions from the two extractions were then combined and evaporated to dryness under a stream of nitrogen gas. The residue was further dried by placing the sample in a vacuum centrifuge (1000 rpm, 0.5 Torr, 25°C) for 18 h. Acetonitrile (50 μl) was added to the residue of the tube, followed by vortex mixing (15 s) and sonication (5 min at 50°C). An aliquot (15 μl) of *N,O*-bis[trimethylsilyl]trifluoro-acetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL) was added to the mixture, which was then mixed by vortex action (15 s), and sonicated (10 min at 50°C). A 1-μl portion of the reaction mixture was injected into the gas chromatograph (GC) for analysis.

2.4. Gas chromatography–mass spectrometry

Analyses were performed using a 5890 Series II gas chromatograph equipped with a capillary inlet and a 5971A Mass Selective Detector, controlled through a DOS-series MS ChemStation (Hewlett-Packard, Palo Alto, CA). Separations were performed on a 15 m×0.25 mm fused-silica capillary column wall-coated with 0.25 μm DB-1 cross-linked

dimethylpolysiloxane (J. & W. Scientific, Folsom, CA). Helium was employed as the carrier gas at a linear velocity of 27.5 cm/s (pentane, 60°C). Temperatures were 285°C at the injection port and 290°C at the transfer line to the detector. Injections were made at an initial oven temperature of 70°C. The oven temperature was held isothermally at 70°C for 2 min, then increased linearly to 280°C at 15°C/min. The final temperature was maintained for 9 min to desorb less volatile components of the sample. Mass spectral detection (electron-ionization, 70 eV) was performed by scanning ions between m/z 50 and 550 at the rate of about 1.5 scan/s. Data were collected between 5 and 25 min postinjection. Nominal resolution and accurate mass measurements were obtained using a VG 70-70 mass spectrometer and data system (Vacuum Generators, Manchester, UK). Samples were introduced into the ion source via either a direct inlet probe or a gas chromatograph which was similarly configured as described above. Accurate mass scans (electron-ionization, 70 eV) were acquired using perfluorokerosene (PFK) as an internal standard.

3. Results and discussion

*3.1. The electron-ionization mass spectrum of trimethylsilylated **2***

Mass spectra of trimethylsilylated compounds are frequently characterized by small or non-existent molecular ions, and prominent M-15 ions resulting from loss of a methyl radical [8,9]. In sharp contrast to this, the electron-ionization (70 eV) mass spectrum of trimethylsilylated **2** (Fig. 2) is strikingly dominated by the molecular ion (m/z 436, base peak, scanning from m/z 50 to 550). An atypical loss of

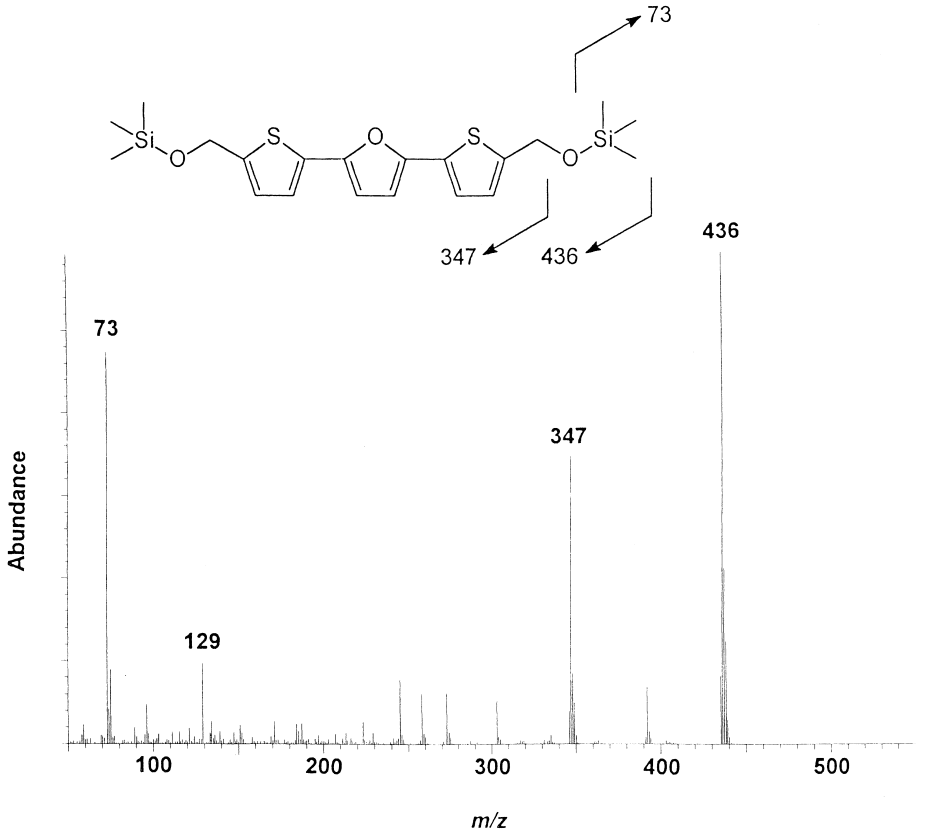


Fig. 2. Electron-ionization (70 eV) mass spectrum of trimethylsilylated **2**.

-*O*-trimethylsilyl (-*O*-TMS (89 amu)) leads to formation of the spectrum's third most abundant ion at *m*/*z* 347 (58%). The propensity for such a loss is undoubtedly enhanced by the molecule's ability to stabilize itself by forming the 2-(*exo*-methylene)-thiophenium group **3** (Fig. 3). That this group may likely be created as a highly electrophilic reaction intermediate (which may in part be responsible for **2**'s observed bioactivity) was demonstrated by separate experiments in which **2**, under mild reaction conditions, was seen to readily form the methyl ether or the ethyl thio-ether in the presence of methanol or ethanethiol, respectively (data not shown).

Except for ions at *m*/*z* 73 (80% (-TMS)), the only other ions in the mass spectrum possessing a relative abundance greater than 15% are observed at *m*/*z* 129 (16%). A ''mass profile scan'' of this region of the mass spectrum shows several partially resolved peaks with apexes at *m*/*z* 129.0, 129.5, 130.0, and 130.5. Furthermore, the abundances of these peaks are proportioned to one another in concordance with values expected for an ion of elemental composition $C_{14}H_{10}OS_2$. Thus, *m*/*z* 129 is a doubly charged species, whose origin can be rationalized by loss of the second -*O*-TMS group (i.e. (436−89−89)/2= 258/2=129).

*3.2. Identification of **2** and its principal metabolite from dog liver S9 incubation*

When incubated with the S9 subcellular fraction of dog liver cells, the concentration of **2** was observed by GC–MS to decline as a function of time, with a concomitant increase in a significant, time-dependent concentration of an unknown entity. Shown in Fig. 4A is the total ion current (TIC) chromatogram resulting from the GC–MS analysis of a sample representing S9 incubation of **2** for 2 h. The ex-

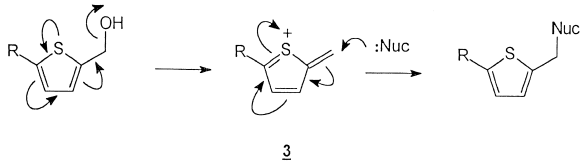


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Fig. 3. Proposed reaction mechanism of the chemical reactivity of **2** towards nucleophiles.

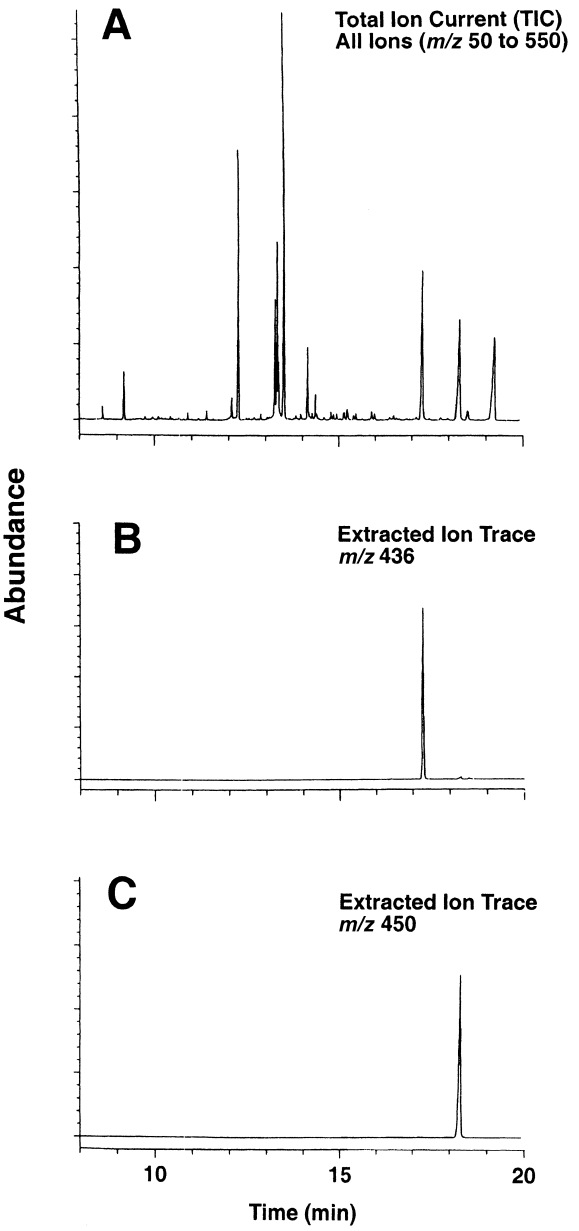

Fig. 4. (A) Total ion current (TIC) chromatogram resulting from GC–MS analysis of an extracted and trimethylsilylated dog liver S9 incubation mixture following 2-h reaction with **2**. (B) Extracted ion chromatogram of *m*/*z* 436 shows one GC peak at 17.3 min, which is identified as trimethylsilylated **2**. (C) The second extracted ion chromatogram of *m*/*z* 450 shows one GC peak at 18.3 min, which is the trimethylsilylated metabolite.

tracted ion chromatogram of *m/z* 436, which is the molecular ion of derivatized (trimethylsilylated) **2**, is seen in Fig. 4B to be unique in the incubation mixture. Analyzed separately, the GC retention time and mass spectrum of a derivatized (trimethylsilylated) authentic sample of **2** shows the 17.3-min GC peak to be **2**.

The area of the 18.3-min GC peak increases as a function of incubation time, and its mass spectrum possesses an abundant high mass ion at *m/z* 450, which is unique in the incubation mixture (see the extracted ion chromatogram of *m/z* 450, depicted in Fig. 4C). The electron-ionization mass spectrum of this GC peak (shown in Fig. 5) has an appearance that is similar to the mass spectrum of the GC peak of **2** at 17.3 min. An abundant *m/z* 450 (base peak, scanning from *m/z* 50 to 550) is most likely the molecular radical cation, and a prominent *m/z* 361 (47%) can be rationalized by the loss of -*O*-TMS. Tentatively assigning *m/z* 450 as the molecular ion of the trimethylsilylated metabolite then allows postulation that S9 incubation of **2** results in a metabolic transformation in which **2** acquires an additional 14 mass units. The most likely transformations to account for this particular change in mass are: (1) methylation (methylene ($-CH_2-$) insertion), or (2) oxidation of one of the hydroxymethyl groups (from $-CH_2OH$ to $-COOH$).

3.3. Preliminary structure elucidation of the metabolite by differential derivatization and accurate mass analysis

Differential derivatization involves the syntheses and analyses of two different derivatives of the same compound. Since the derivatives differ in mass, the number of derivatizable groups of the original compound may be deduced. If, for example, a simple alcohol is converted into the corresponding trimethylsilyl ether, then 72 mass units have been added to the m.w. of the original alcohol. If another sample of that alcohol is converted into the corresponding *tert.*-butyl dimethylsilyl ether, then 114 mass units have been added to the m.w. of the original alcohol. The difference in m.w. between these two derivatives is 42 mass units. Thus, the task of determining the number of alcohol groups in a compound may be approached by separately synthesizing and analyzing the trimethylsilyl ether and the *tert.*-butyl dimethylsilyl ether derivatives. If the

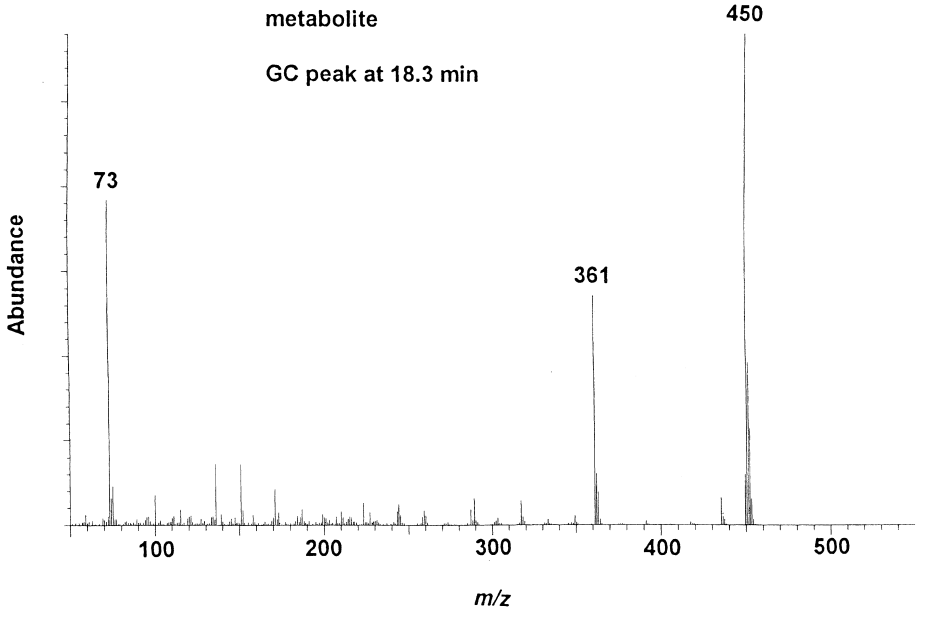

Fig. 5. Electron-ionization (70 eV) mass spectrum of the trimethylsilylated metabolite.

analyses show that the m.w. of the two ethers differ by 42 mass units, then the original compound possesses one alcohol group; if the analysis shows that the m.w. of the two ethers differ by 84 mass units, then the original compound possesses two alcohol groups, and so on.

Thus, **2** was subjected to differential derivatization using trimethylsilylation and *tert.*-butyl dimethylsilylation reagents. GC–MS analyses of the trimethylsilylated and *tert.*-butyl dimethylsilylated derivatives indicate a m.w. of 436 for the former and 520 for the latter. The mass difference of 84 supports the notion that **2** possesses two derivatizable (i.e. alcohol) groups.

Similarly, the metabolite was subjected to the same differential derivatization: GC–MS analyses of the trimethylsilylated and *tert.*-butyl dimethylsilylated derivatives indicate a m.w. of 450 for the former and 534 for the latter. The mass difference of 84 is indicative of the metabolite possessing two derivatizable groups. Although many functional groups can be silylated [9], the metabolite most likely possesses alcohol and/or carboxylic acid groups.

If the formation of the metabolite is due to methylation (addition of $-CH_2-$), the expected accurate mass is 450.1175 (calculated for $C_{21}H_{30}O_3S_2Si_2$). If, however, the formation of the metabolite is due to oxidation, the expected accurate mass is 450.0811 (calculated for $C_{20}H_{26}O_4S_2Si_2$). The accurate mass analysis of the trimethylsilylated metabolite was performed (found: 450.0749), the results of which verify a molecular formula concordant with metabolic transformation of one of the two hydroxymethyl groups of **2** into a carboxylic acid functionality (Fig. 6).

*3.4. Oxidation of the mono-aldehydic analog of **2** and GC–MS comparison with the metabolite*

Chemical treatment of an authentic sample of the mono-aldehydic analog of **2** (5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxaldehyde (NSC 672170)) with basic silver oxide [10,11] produced a simple mixture. The most abundant component of the mixture, after trimethylsilylation, possessed a GC retention time (18.3 min) and mass spectrum that were identical to those of the metabolite.

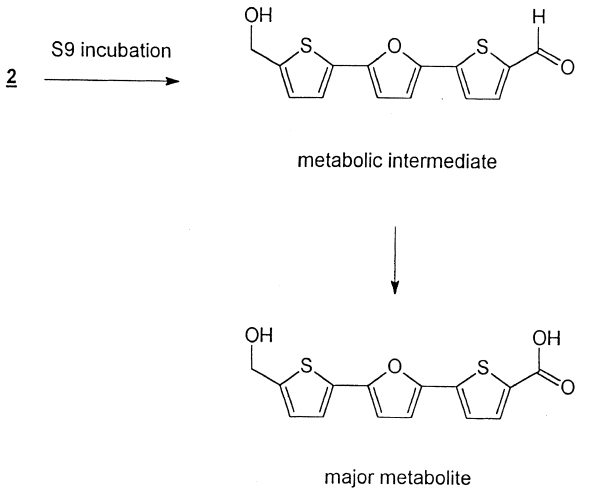

Fig. 6. Proposed metabolic pathway and chemical structure of the major metabolite resulting from dog liver S9 incubation of **2**.

*3.5. Dog liver S9 incubation of the mono-aldehydic analog of **2***

In a separate experiment, an authentic sample of the mono-aldehydic analog of **2** (5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxaldehyde (NSC 672170)) was incubated with dog liver S9 for 2 h and then processed and analyzed by GC–MS as before. Although little of the aldehyde remained, its presence was nonetheless confirmed by its GC retention time and mass spectrum. However, a large GC peak was identified by its GC retention time and mass spectrum to be **2**, and an even larger GC peak possessed a GC retention time and mass spectrum identical to those of the S9 metabolite of **2**. Since oxidation of primary alcohols to aldehydes is reversible, it should not be unexpected that the metabolism of aldehydes would likely result in formation of both the corresponding alcohol and carboxylic acid [12].

4. Conclusions

Incubation of **2** with dog liver S9 results in the formation of one major metabolite, which has been

identified by mass spectrometric methods as 5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxylic acid. Separate incubation of 5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxaldehyde with dog liver S9 resulted in a mixture of predominantly **2** and 5-[5-[5-hydroxymethyl-2-thienyl]-2-furanyl]-2-thiophenecarboxylic acid, thereby suggesting the involvement of alcohol dehydrogenase in the metabolism of **2**. The metabolic pathway of **2** is most likely a two-step oxidation process, with transformation of the hydroxymethyl group to a carboxylic acid moiety via a carboxaldehyde.

Acknowledgements

This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

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