

Antiproliferative Activity of Derivatives of *trans*-Bis(salicylaldoximato)copper(II) *in vitro*. Some *in vivo* Properties of the Parent Compound

Hannu Elo and Paavo Lumme

Division of Inorganic Chemistry, Department of Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki 10, Finland

Z. Naturforsch. **41c**, 951–955 (1986);
received March 7/May 15, 1986

Copper Chelates, Antiproliferative Activity, Pyridoxal Hydrochloride, Toxicity, L1210 Leukemia Cell Culture

Several derivatives and analogs of the recently reported antiproliferative and antitumor agent *trans*-bis(salicylaldoximato)copper(II) (CuSAO_2) have been prepared and tested for antiproliferative activity against L1210 leukemia cells *in vitro*. The salicylaldimine analog of CuSAO_2 had a very strong antiproliferative activity, the 2-day IC_{50} value being lower than 3 $\mu\text{g}/\text{ml}$. The 2,3-dihydroxybenzaloxime analog was equally active with CuSAO_2 , while the corresponding 2,5-dihydroxy derivative had a slightly lower activity. The 2,3,4-trihydroxybenzaloxime derivative had a much lower activity than had the dihydroxybenzaloxime derivatives. The zinc(II) analog of CuSAO_2 had only a low antiproliferative activity. The ligand of CuSAO_2 , salicylaldoxime, resembles pyridoxal oxime, a vitamin B_6 antagonist and a powerful inhibitor of pyridoxal kinase. An attempt to reduce the toxicity of CuSAO_2 *in vivo* with pyridoxal hydrochloride led to increased toxicity.

Introduction

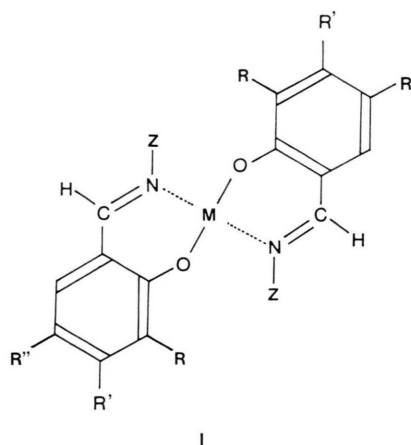
We have recently shown that *trans*-bis(salicylaldoximato)copper(II) (CuSAO_2 ; **Ia**, $\text{Z} = \text{OH}$, $\text{M} = \text{Cu}$, $\text{R} = \text{R}' = \text{R}'' = \text{H}$) and its derivative *trans*-bis(2,4-dihydroxybenzaloximato)copper(II) (CuRES_2 ; **Ib**, $\text{Z} = \text{OH}$, $\text{M} = \text{Cu}$, $\text{R} = \text{R}'' = \text{H}$, $\text{R}' = \text{OH}$) have a very strong antiproliferative activity against tumor cells *in vitro*. Both compounds were shown to totally arrest the proliferation of leukemia L1210 and Ehrlich ascites carcinoma cells in the concentration of about 5 $\mu\text{g}/\text{ml}$ [1]. The antiproliferative activity is a property of the intact chelates only. The ligand part of CuSAO_2 , salicylaldoxime, had no antiproliferative activity even in high concentrations, and free copper(II) salts had only a very limited activity [1].

Further, it was shown that CuSAO_2 has strong antitumor activity against Ehrlich ascites carcinoma

Abbreviations: CuRES_2 , *trans*-bis(2,4-dihydroxybenzaloximato)copper(II), also known as *trans*-bis(β -resorcyldaldoximato)copper(II); CuSAO_2 , *trans*-bis(salicylaldoximato)copper(II); EAC, Ehrlich ascites carcinoma.

Reprint requests to H. Elo.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0900–0951 \$ 01.30/0



in mice *in vivo* [2]. Because of these promising results, further studies on the compounds and their derivatives are warranted. Screening of new derivatives is especially interesting, not only because it may lead to the discovery of new active compounds, but also because it may reveal possible structure-activity correlations. Such correlations may in turn be valuable in the elucidation of the mechanism of action of the compounds, and may also form the basis for a more rational search of derivatives of higher activity. Therefore, we have now synthesized some derivatives and analogs of CuSAO_2 and CuRES_2 , and tested them against leukemia L1210 cells *in vitro*. We also report here the results of some preliminary *in vivo* experiments on CuSAO_2 .

Materials and Methods

Chemicals

CuSAO_2 [3] and the corresponding Zn(II) chelate (**Ic**) [4] were prepared as previously described. Compound **Id** (see Table I for substituents) was prepared according to the method of Pfeiffer *et al.* [5]. Compound **II** was prepared essentially according to the method of Dubský and Sokol [6]. The syntheses of other compounds will be described elsewhere. Analytical data on the compounds tested *in vitro* are given in Table I.

Pyridoxal hydrochloride (biochemical grade) and dimethyl sulfoxide (spectroscopical grade or scintillation grade) were obtained from E. Merck.

In vitro screening

Mouse L1210 leukemia cells were grown at 37 °C in Gibco's medium RPMI 1640 supplemented with



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

5% (v/v) of pooled human serum (Finnish Red Cross Transfusion Service), 2 mM glutamine, and 50 mg of the sodium salt of penicillin G and 50 mg of streptomycin per litre. The cells were counted using a particle counter (Coulter Electronics, model Industrial D). — For each test series, a confluent cell suspension was diluted, the cells were counted, and 5 ml aliquots of the suspension were grown in tightly closed disposable culture tubes of 10 ml volume. The test substances were added as solutions or suspensions in dimethyl sulfoxide (E. Merck) (25 µl/5 ml). Controls and solvent controls were employed. The number of cells in each tube was counted after 1 and 2 days. For each compound and concentration tested, the value of the growth ratio

$$Y = \frac{N - A}{C - A}$$

was calculated, where N is the number of cells in the tube containing the test substance, C is the mean number of cells in the solvent control tubes, and A is the initial value. Percentage inhibition, obtained by subtracting the value of Y from 100%, was plotted against concentration, and approximate concentrations which inhibit growth to 50% of control growth (IC_{50}) were determined graphically.

In vivo experiments

NMRI mice were obtained from the Department of Pharmacy of the University of Helsinki. They were housed at ordinary room temperature with a

12 h light-dark cycle and were given a standard laboratory diet and tap water *ad libitum*.

The detergent solution used in the pyridoxal experiment was prepared by dissolving 100 µl of Tween 20® (polyoxyethylenesorbitan monolaurate; Ph. Eur. grade) in 100 ml of 0.9% aqueous NaCl solution.

Results and Discussion

In vitro screening of derivatives of CuSAO₂

Most of the CuSAO₂ derivatives and analogs tested had the general formula **I**. Table I shows the results of screening in cell culture. Usually, four different concentrations were tested for each compound.

The salicylaldehyde analog (**Ic**) of CuSAO₂ was found to have a considerable antiproliferative activity. During the first day, its IC_{50} value was as low as ca. 3–4 µg/ml, the 2-day value being even lower. The compound is a potential candidate for testing *in vivo*. The results given in Table I were obtained with a freshly prepared solution of **Ic**. The compound is unstable in dimethyl sulfoxide, as indicated by color changes, and when the solutions are allowed to stand for several days, the antiproliferative activity is lost almost totally. The corresponding zinc chelate (**Id**) had a much weaker activity.

We have previously shown that the nickel(II) analog of CuSAO₂ has only a very low antiproliferative activity, while the activity of the cobalt(II) analog is almost as high as that of CuSAO₂ [1]. The

Table I. Results of screening of derivatives and analogs of CuSAO₂ against leukemia L1210 cells *in vitro*^a.

Com-pound	Structure						IC ₅₀ [μg/ml]		Formula	Analytical data				Found [%]				
		Z	M	R	R'	R''	1 day	2 days		Calculated [%]			Cu	C	H	N	Cu	
										C	H	N	Cu	C	H	N	Cu	
Ic	H	Cu	H	H	H	3–4	< 3	Cu(C ₇ H ₆ NO) ₂					9.22	20.9			9.09	20.5
Id	H	Zn	H	H	H	10–20	5–10	Zn(C ₇ H ₆ NO) ₂										
Ie^b	OH	Zn	H	H	H	5–20	> 20	Zn(C ₇ H ₆ NO ₂) ₂ · H ₂ O										
If	OH	Cu	OH	H	H	4	4	Cu(C ₇ H ₆ NO ₃) ₂	45.72	3.29				45.05	3.21			
Ig	OH	Cu	H	H	OH	2.5–5	2.5–5	Cu(C ₇ H ₆ NO ₃) ₂ · H ₂ O	43.58	3.66				43.61	3.60			
Ih	OH	Cu	OH	OH	H	18	10	Cu(C ₇ H ₆ NO ₄) ₂	42.06	3.03				41.47	3.00			
Ii	(CH ₂) ₂ OH	Cu	H	H	H	17–50	9	Cu(C ₉ H ₁₀ NO ₂) ₂	55.16	5.14	7.15	16.2	55.86	5.20	6.84		15.2	
Ij	(CH ₂) ₃ OH	Cu	H	H	H	10–20	< 9	Cu(C ₁₀ H ₁₂ NO ₂) ₂				15.1						15.0
Ik	(CH ₂) ₃ OH	Cu	OH	H	H	5–10	12	Cu(C ₁₀ H ₁₂ NO ₃) ₂ · H ₂ O	51.11	5.58				50.71	5.42			
Il						> 20	> 20	Cu(C ₁₆ H ₁₄ N ₂ O ₂)	58.26	4.28	8.50	19.3	58.50	4.36	8.16		18.02	

^a The IC_{50} values should be considered as rough estimates only. Determination of accurate values was not deemed important for the purposes of this study. The IC_{50} values of CuSAO₂ and CuRES₂ are between 3 and 4 µg/ml.

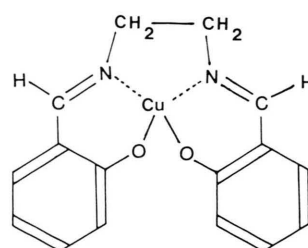
^b This compound was added to the cell culture as a suspension.

present results indicate that the corresponding zinc(II) chelate (**Ie**) is only a weak inhibitor of tumor cell proliferation. Copper and cobalt compounds are usually more prone to redox reactions than are nickel and zinc compounds. Thus, taken together, the results suggest that redox reactions may have a role in the antiproliferative activity of CuSAO₂ and related compounds. Other explanations for the low activity of the nickel and zinc chelates cannot, however, be ruled out. For example, the low activity of the zinc chelate may at least in part be due to its very low solubility.

The 2,3- and 2,5-dihydroxybenzaldehyde derivatives (**If**) and (**Ig**) are isomers of the previously studied CuRES₂ [1]. All of these compounds have a very high antiproliferative activity against L1210 leukemia cells. The *in vitro* dose-response curves of CuRES₂ [7] and **If** rise very steeply between 3 and 5 µg/ml, just like that of CuSAO₂ [7], and reach ca. 90% inhibition at about 5 µg/ml. In the case of **Ig**, however, the rise of the curve is somewhat less steep, and higher concentrations are needed for 90% inhibition. The antiproliferative activity of the 2,3,4-trihydroxybenzaldehyde derivative (**Ih**) is clearly lower than that of the dihydroxybenzaldehyde derivatives studied. Even at the concentration of 20 µg/ml, only 60% inhibition was achieved with this compound.

Hodnett and coworkers have shown that certain cobalt(II) complexes of Schiff bases of salicylaldehyde and aminoalcohols have antitumor activity [8–10]. The chelate of the Schiff base of salicylaldehyde and 3-amino-1-propanol appeared to have especially good antitumor activity [8]. Also some related Schiff bases in their own right slow tumor growth [10–14]. The cobalt complexes reported by Hodnett *et al.* are structurally related to CuSAO₂. Therefore, we prepared the copper(II) chelates (**Ii**) and (**Ij**) of the salicylaldehyde Schiff bases of 2-amino-1-ethanol and 3-amino-1-propanol, respectively. The chelate **Ik** of the Schiff base formed by 2,3-dihydroxybenzaldehyde and 3-amino-1-propanol was also synthesized. All of these compounds had moderate antiproliferative activity but were clearly less active than CuSAO₂.

Compound **II**, the copper(II) chelate of the tetradentate ligand N,N'-bis(salicylidene)ethylenediamine, was the only one of the present CuSAO₂ analogs that did not have any measurable antiproliferative activity. There is at least one major difference between the structure of this compound and all



II

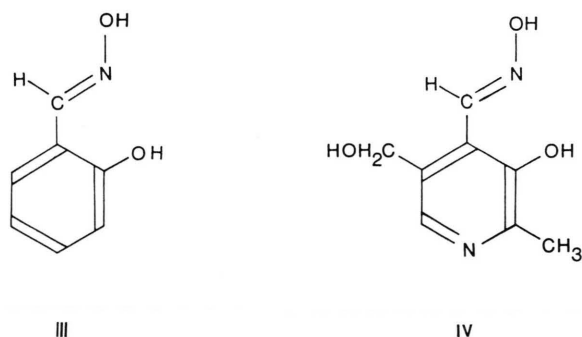
the others tested. Namely, compound **II** cannot exist in the *trans*-configuration that is typical of most CuSAO₂ type compounds. This configurational restriction, caused by the structure of the ligand, may well lie behind the total lack of antiproliferative activity. We are currently engaged in the synthesis of other analogs having a similar configurational restriction. Studies on them might reveal, whether *trans*-configuration about the metal ion is essential for the antiproliferative activity of CuSAO₂ type compounds. This point is very interesting, because in the case of the well-known and clinically important platinum anticancer drugs, the *cis*-isomers have high anti-tumor activity while the *trans*-isomers do not show any appreciable activity [15–17]. Hall and Waters [18] have shown that the configuration about the copper atom in **II** is pyramidal in the solid state. It is possible that the compound has this configuration in solution, too. Such non-planarity might also lie behind the lack of biological activity.

In vivo studies on CuSAO₂

We have previously reported [2] experiments showing that CuSAO₂ has a remarkable antitumor activity against EAC *in vivo*. In those experiments, some CuSAO₂-treated mice were totally cured, being still alive and apparently healthy 101 days after the injection of EAC cells. Four of those mice were then used for a preliminary immunity test, the results of which are reported here. Each of the test mice (weighing 28–35 g) and each of the ten control mice (weighing 24–34 g, mean 29.7 ± 2.58 g) were given i.p. 0.18 × 10⁶ EAC cells. The mean survival time of the controls was 22.1 ± 2.2 days. Two of the cured CuSAO₂-treated mice died of EAC (survival times 20 and 21 days). The other two were resistant and did not take EAC. No increase in their body weights was

detected during 40 days. These very preliminary results are in line with those of Kimoto and coworkers [19], who treated EAC-inoculated mice with a combination of sodium ascorbate and the copper(II) chelate of glycylglycylhistidine and found that those mice that survived over 60 days, were resistant to a new EAC inoculum. Therefore, further studies on the immunological consequences of treatment of tumors with CuSAO_2 and other Cu(II) compounds are strongly warranted and might give valuable information on the mechanisms of tumor immunity.

The ligand of CuSAO_2 , salicylaldoxime (**III**), resembles pyridoxal oxime (**IV**), a well-known vitamin



B_6 analog and antagonist and an extremely powerful inhibitor of pyridoxal kinase [20–23]. On the other hand, vitamin B_6 deficiency and B_6 antagonists are known to have growth inhibiting and antitumor activity [24, 25]. These data, together with the finding that the side-effects of CuSAO_2 (acrodynia and weight loss) [2] resemble those of vitamin B_6 deficiency and of B_6 antagonists [24, 26], led us to explore the possible interaction of CuSAO_2 and the B_6 vitamer pyridoxal. It was hoped that pyridoxal could be used as an antidote of CuSAO_2 .

Thus, a preliminary study was performed, in which female NMRI mice were given i.p. either first saline and 30 minutes thereafter CuSAO_2 (30 mg kg^{-1}), or first pyridoxal hydrochloride (100 mg kg^{-1}) and then CuSAO_2 (30 mg kg^{-1}), or first pyridoxal hydrochloride (100 mg kg^{-1}) and then saline. The treatments were repeated after 7 h. Appropriate vehicle controls were also employed. (For details, see the legend of Fig. 1.) As is evident from Fig. 1, the mice which twice received pyridoxal hydrochloride and CuSAO_2 , suffered a weight loss approximately equal to that of the animals which twice received saline and CuSAO_2 , but recovered weight more slowly than the latter. Thus, in the dosage used, pyridoxal hydro-

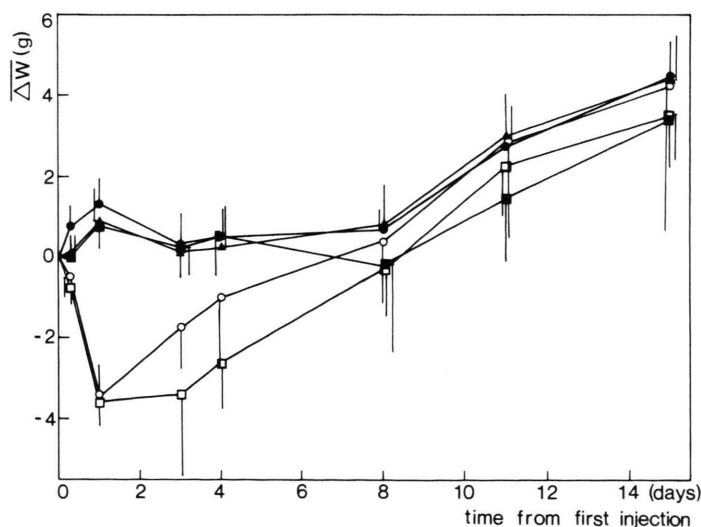


Fig. 1. Mean changes of body weight (ΔW) of mice of different groups in the pyridoxal hydrochloride- CuSAO_2 test, as compared to the corresponding weights just before the first injection. Female NMRI mice weighing $23.7 \pm 4.4 \text{ g}$ were used, divided into five groups of six animals. At the beginning of the experiment, groups I–III were given i.p. 0.9% NaCl solution (0.1 ml/10 g body weight), and 30 min thereafter a second i.p. injection of the same volume was given, group I (●) receiving again 0.9% NaCl solution, group II (▲) receiving the detergent solution (see Materials and Methods) and group III (○) receiving a suspension of CuSAO_2 in the detergent solution (30 mg/10 ml, thus 30 mg per kg body weight). Groups IV (□) and V (■) received at the beginning of the experiment i.p. 0.9% NaCl solution containing 1% (w/v) pyridoxal hydrochloride (injection volume 0.1 ml/10 g body weight, thus 100 mg pyridoxal hydrochloride per kg), and 30 min thereafter group IV received CuSAO_2 (30 mg/kg body weight) in the detergent solution (0.1 ml/10 g) and group V received 0.9% NaCl solution (0.1 ml/10 g). The first injections were repeated 7 hours after the beginning of the experiment, and the second injections 30 min later. Thereafter no treatments were done. — The bars indicate standard deviations.

chloride did not reduce but, if anything, increased the toxicity of CuSAO₂. The administration of saline and pyridoxal hydrochloride did not cause any weight loss. Two of the six mice treated with pyridoxal hydrochloride and CuSAO₂ died (during days 3 and 4), while all mice in other groups survived to the end of the experiment (21 days).

In spite of the finding that pyridoxal did not reduce the toxicity of CuSAO₂, the possibility cannot be excluded that the mechanism of action of CuSAO₂ is based on vitamin B₆ antagonism. B₆ antagonists are namely known, whose effects cannot be reversed by B₆ vitamers [27]. Thus, further studies on the possible role of pyridoxal kinase inhibition in the mechanism of action of CuSAO₂ type compounds are war-

ranted. An experiment of the type described above but employing various different timing and dosage schedules might also be warranted.

Acknowledgements

Miss Raija Laine, Mr. Jarmo Pajunen, Miss Gun-Susanne Roth, and Mr. Ehsan Etemadzadeh, M. Sc., are acknowledged for skilful technical assistance. Professor Liisa Ahtee and Associate Professor Juhani Jänne are acknowledged for their cooperation.

H. E. has been financially supported by a grant from the Kymenlaakson rahasto, a regional fund of the Finnish Cultural Foundation.

- [1] P. Lumme, H. Elo, and J. Jänne, *Inorg. Chim. Acta* **92**, 241–251 (1984).
- [2] H. O. Elo and P. O. Lumme, *Cancer Treat. Rep.* **69**, 1021–1022 (1985).
- [3] P. Lumme and M.-L. Korvola, *Thermochim. Acta* **13**, 419–439 (1975).
- [4] P. Lumme, *Suomen Kemistilehti* **32B**, 261–265 (1959).
- [5] P. Pfeiffer, E. Buchholz, and O. Bauer, *J. Prakt. Chem.* **129**, 163–177 (1931).
- [6] J. V. Dubský and A. Sokol, *Coll. Czech. Chem. Commun.* **3**, 548–549 (1931); *cf. Chem. Abstr.* **26**, 1538 (1932).
- [7] P. O. Lumme and H. O. Elo, *Inorg. Chim. Acta* **107**, L15–L16 (1985).
- [8] E. M. Hodnett and W. J. Dunn, *J. Med. Chem.* **15**, 339 (1972).
- [9] E. M. Hodnett, C. H. Moore, and F. A. French, *J. Med. Chem.* **14**, 1121–1123 (1971).
- [10] E. M. Hodnett and W. Willie, *Proceedings of the Oklahoma Academy of Sciences* **46**, 107–111 (1965).
- [11] E. M. Hodnett and W. J. Dunn, *J. Med. Chem.* **13**, 768–770 (1970).
- [12] E. M. Hodnett and P. D. Mooney, *J. Med. Chem.* **13**, 786 (1970).
- [13] J. H. Billman, F. Koehler, and R. May, *J. Pharm. Sci.* **58**, 767–769 (1969).
- [14] J. D. Modi, S. S. Sabnis, and C. W. Deliwala, *J. Med. Chem.* **13**, 935–941 (1970).
- [15] B. Rosenberg, L. VanCamp, J. E. Trosko, and V. H. Mansour, *Nature (London)* **222**, 385–386 (1969).
- [16] M. J. Cleare and P. C. Hydes, *Antitumor properties of metal complexes*, in: *Metal Ions in Biological Systems* (H. Sigel, ed.), **Vol. 11**, p. 1–62, Marcel Dekker, New York 1980.
- [17] B. Rosenberg, *Clinical aspects of platinum anticancer drugs*, in: *Metal Ions in Biological Systems* (H. Sigel, ed.), **Vol. 11**, p. 127–196, Marcel Dekker, New York 1980.
- [18] D. Hall and T. N. Waters, *J. Chem. Soc.* **1960**, 2644–2648.
- [19] E. Kimoto, H. Tanaka, J. Gytoku, F. Morishige, and L. Pauling, *Cancer Res.* **43**, 824–828 (1983).
- [20] D. B. McCormick and E. E. Snell, *J. Biol. Chem.* **236**, 2085–2088 (1961).
- [21] D. B. McCormick and E. E. Snell, *Methods Enzymol.* **18A**, 611–619 (1970).
- [22] F. Kwok and J. E. Churchich, *J. Biol. Chem.* **254**, 6489–6495 (1979).
- [23] J. E. Churchich and C. Wu, *J. Biol. Chem.* **256**, 780–784 (1981).
- [24] F. Rosen, E. Mihich, and C. A. Nichol, *Vitam. Horm.* **22**, 609–641 (1964).
- [25] W. Korytnyk and M. Ikawa, *Methods Enzymol.* **18A**, 524–566 (1970).
- [26] G. A. Emerson, *Fed. Proc.* **6**, 406–407 (1947).
- [27] W. Korytnyk, *Methods Enzymol.* **62D**, 454–483 (1979).