CHROM. 4253

CHROMATOGRAPHIC PROPERTIES OF SOME CYCLIC α-IMINO ACIDS HOMOLOGOUS TO PROLINE, AND THEIR DNP-, DNS- AND PTH-DERIVATIVES

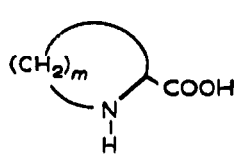
H. T. NAGASAWA, P. S. FRASER AND J. A. ELBERLING
Cancer Research Laboratory, Minneapolis Veterans Hospital and the Department of Medicinal Chemistry, Universtiy of Minnesota, Minneapolis, Minn. 55417 (U.S.A.)
(Received June 27th, 1969)

SUMMARY

The chromatographic behavior of the homologous cyclic α-imino acids of ring sizes ranging from four- to eleven-members (including proline) on ion-exchange and thin-layer systems is described. The validity of the Martin relation in the homologous series was verified. This was further cogently demonstrated by the "non-fit" of the isomeric but non-homologous α-imino acid 4,4 dimethylproline. The 2,4-dinitrophenyl- (DNP-), 5-dimethylaminonaphthalenesulfonyl- (DNS-), and phenylthiohydantoin- (PTH-) derivatives of these imino acids were also separated by TLC. Within certain limitations, the Martin derivation also appeared to be linearly related to the number of methylene groups in some of these derivatives.

INTRODUCTION

We have recently described[1] the synthesis of a homologous series of α-imino acids (*1d–1i*) which represent the higher membered ring homologs of L-proline (*1c*), the naturally occurring α-imino acid of protein origin. Whereas the occurrence in the plant kingdom of the six-membered pipecolic acid (*1d*) and its analogs have been adequately documented[2–4], the possible presence in nature of the homologous medium ring compounds, namely hexahydroazepine-2- carboxylic acid (*1e*), octahydroazocine-



1a $m = 1$ *1f*, $m = 6$
b, $m = 2$ *g*, $m = 7$
c, $m = 3$ *h*, $m = 8$
d, $m = 4$ *i*, $m = 9$
e, $m = 5$

2-carboxylic acid (*1f*), octahydroazonine-2-carboxylic acid (*1g*), decahydroazecine-2-carboxylic acid (*1h*), and azacycloundecane-2-carboxylic acid (*1i*), respectively the

seven-through-eleven-membered cyclic α-imino acids, has not been investigated, although a rational biochemical basis for their occurrence cannot presently be advanced.

The very first member* of this cyclic α-imino acid series, aziridine-2-carboxylic acid (*1a*), is strictly a synthetic chemical entity known to be unstable and therefore not isolable except as the ester or as salts[5,6]. The next higher homolog, L-azetidine-2-carboxylic acid (*1b*), is relatively stable, has been isolated from, and constitutes the major soluble nitrogenous nonprotein component of the Liliacae[7–9]. *1b*, a proline antagonist which inhibits protein synthesis, substitutes for L-proline in the protein of *Escherichia coli* and of the seedlings of the mung bean, *Phaseolus aureus*[10]. *1b* is also incorporated into the actinomycin molecule when added to the culture medium of *Streptomyces antibioticus* or of *S. chrysomallus* to give new biosynthetic actinomycins[11].

The possibility that α-imino acids of medium ring size might occur as natural constituents or metabolites of plants or of other organisms does not appear so tenuous if one considers the wide variety of structurally unique amino acids of non-protein origin that have been discovered in recent years[2–4]. For example, the imino acid of unknown structure recently isolated by BLUNDEN AND CHALLEN[12] from *Salix fragilis* leaf galls might possibly correspond to one of these in our series, although additional evidence is obviously required. In order to facilitate their detection and identification, if and when their presence is suspected, we have prepared a number of derivatives of these homologous α-imino acids, namely, the DNP-, DNS- and PTH-derivatives, and herewith record their chromatographic properties in TLC systems. The chromatographic properties of the parent α-imino acids are also described.

EXPERIMENTAL

The DNP- and DNS-derivatives of the imino acids were prepared by slight modifications of procedures described in standard reference works[13,14]; however, the preparative procedure for the imino acid phenylthiohydantoins differed from the usual methods. 4,4-Dimethyl-DL-proline, a proline analog where the 4-position (the position where proline, as peptidyl proline, is usually hydroxylated) is blocked by methyl groups, was also synthesized in the course of this work**. L-Azetidine-2-carboxylic acid, L-proline, the latter's DNP- and DNS-derivatives, and L- and DL-pipecolic acids were purchased from Calbiochem (Los Angeles, Calif.), Nutritional Biochemicals Corp. (Cleveland, Ohio), or Aldrich Chemical Co. (Milwaukee, Wisc.).

Paper chromatography was accomplished on Whatman No. 1 paper using the descending technique. The availability of commercial pre-coated TLC plates dictated their selection for thin-layer chromatography in order that others wishing to duplicate our results would not be faced with quality differences of homemade plates as they may exist in different laboratories. However, laboratory-coated TLC plates were also used. Selection of solvent systems and adsorbants were based on (a) their ability to separate each member of the homologous series, and (b) where possible, previous history of their use for separation and identification of amino acids in protein hydroly-

* Glyoxalimine (*1*, $m = 0$), theoretically the lowest member of this series, is not a ring system in the classical sense and will not be considered.

** The description of these procedures as well as certain other analytical data will be presented elsewhere.

sates[14,15], in consideration of possible future application for contemplated biochemical studies. For ion-exchange chromatography of the free α-imino acids a Beckman-Spinco Model 120 Amino Acid Analyzer, column size 0.9 × 63 cm with AA-15 resin, flow rate 18.6 ml/h was used. The sodium citrate buffers were of pH 3.28 (± 0.01) and 4.25 (± 0.01), and the buffer change was programmed to take place at 90 min. The mixture applied contained 1 μmole of each imino acid and 0.1 μmole each of the internal markers, aspartic acid, glycine and tyrosine, so that a direct visual comparison of the relative extinctions of the ninhydrin generated chromophores at 440 and 570 mμ could be made by inspection. For visualization of the free α-imino acids, the paper or thin-layer chromatograms were sprayed in the usual manner with 0.3 % ethanolic ninhydrin reagent which is available commercially in aerosol dispensers. Proline and dimethylproline gave yellow colors, and pipecolic acid the usual violet color. All the others in the homologous series gave mauve colors, although the seven membered *1e* had a somewhat yellowish tinge. The DNP-derivatives were all visible as yellow spots (they were also fluorescencequenching when the TLC adsorbant also contained a UV fluorescing background), while the DNS-derivatives all fluoresced bright yellow. The PTH-derivatives were visualized under a UV light source by quenching of the fluorescent background. The limits of detectability were of the same order of magnitude reported for the DNP-, DNS and PTH-derivatives of other amino acids[14].

RESULTS AND DISCUSSION

Chromatographic separation of the free α-imino acids

Fig. 1 shows the elution profile of the homologous series of cyclic α-imino acids from the 4-membered L-azetidine-2-carboxylic acid (*1a*) to the 11-membered azacycloundecane-2-carboxylic acid (*1i*) from an ion-exchange column. Not surprisingly, the imino acids were eluted in the order of increasing molecular weights. The positions of the individual imino acids were checked by subjecting each one, singly, to ion-

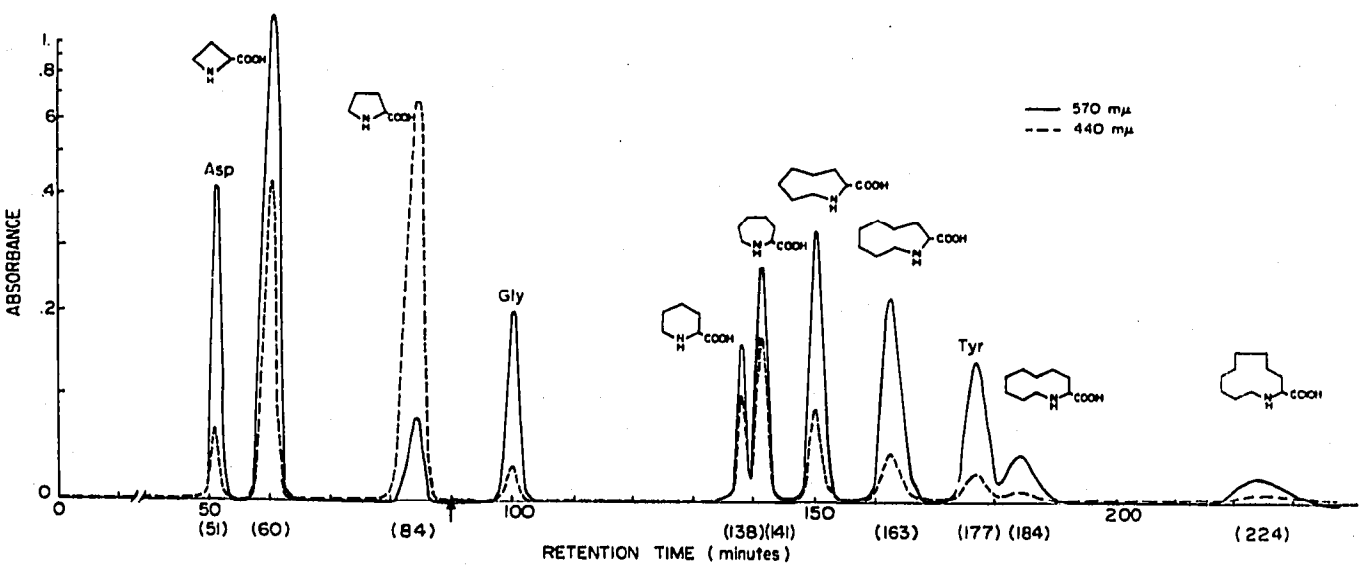


Fig. 1. Chromatographic behavior of the homologous series of cyclic α-imino acids containing four to eleven members in the ring on an ion-exchange column. Their retention times can be compared to that of the marker amino acids, aspartic acid, glycine and tyrosine. Buffer change at the arrow.

exchange chromatography. No overlaps or crossovers were noted. The considerable lag in the elution time with concomitant broadening of the peak of the highest molecular weight member of this series, *viz.*, azacycloundecane-2-carboxylic acid (*1i*), is due largely to its molecular size and lipophilicity since its p*K*a (10.1) is not much different from that of the 9-membered octahydroazonine-2-carboxylic acid (*1g*, p*K*a 10.5). The marker amino acids, aspartic acid, glycine and tyrosine served to orient the positions of some of the naturally occurring amino acids to the elution profile of the cyclic imino acids.

A number of paper and TLC systems were used to separate this homologous series of α-imino acids, and these are recorded in Table I. The relatively long periods required for a run (up to 18 h) and the observed broadening of the applied spots make paper chromatographic separations less appealing than the TLC method.

TABLE I

SEPARATION OF HOMOLOGOUS CYCLIC α-IMINO ACIDS BY PAPER AND THIN-LAYER CHROMATOGRAPHY

Paper chromatography on Whatman No. 1 paper; TLC on Silica Gel HF_{254}, laboratory-coated (solvent F), or precoated Silica Gel F_{254} plates (E. Merck, A.G., distributed by Brinkmann Instruments Inc., Westbury, N.Y.) (solvent G). Solvents: A = acetic acid–*tert.*-amyl alcohol–H_2O (1:10:2); B = *tert.*-amyl alcohol sat'd with H_2O; C = butyl acetate–ethanol–triethylamine–H_2O (5:5:2:3); D = 2-butanone–*n*-butyl alcohol–triethylamine–H_2O (10:10:2:5); E = 2,6-lutidine–*tert.*-amyl alcohol sat'd with H_2O (1:1) (ref. 16); F = *n*-butyl alcohol–acetic acid–H_2O (4:1:1); G = *n*-propyl alcohol–H_2O (7:3). Development time: 18 h for solvents A, B and E; 7 h for solvent C; 6 h for solvent D; ~3 h for solvents F and G.

Number of methylene groups (m)	*α-Imino acid*	*R_F values in solvents*						
		Paper chromatography					*TLC*	
		A	*B*	*C*	*D*	*E*	*F*[a]	*G*[b]
2	L-Azetidine-2-carboxylic acid	0.05	0.04	0.13	0.05	0.16	0.15	0.30
3	L-Proline	0.09	0.07	0.19	0.06	0.23	0.17	0.36
4	DL-Pipecolic acid	0.15	0.13	0.28	0.10	0.29	0.22	0.43
5	Hexahydroazepine-2-carboxylic acid	0.23	0.20	0.34	0.17	0.39	0.28	0.51
6	Octahydroazocine-2-carboxylic acid	0.32	0.28	0.42	0.27	0.46	0.33	0.56
7	Octahydroazonine-2-carboxylic acid	0.40	0.39	0.52	0.42	0.55	0.39	0.62
8	Decahydroazecine-2-carboxylic acid	0.46	0.49	0.69	0.64	0.63	0.45	0.67
9	Azacycloundecane-2-carboxylic acid	0.56	0.57	0.75	0.73	0.70	0.52	0.72
4	4,4-Dimethyl-DL-proline	—	—	—	—	—	—	0.55[b]

[a] Average of 6 determinations S.D. ≤ 0.02
[b] Average of 12 determinations. S.D. ≤ 0.02.

The presence of a unique ring homology here prompted us to evaluate the validity of the Martin relation[14] in this series of α-imino acids. As can be seen from Fig. 2, the Martin derivation, R_M*, was indeed a linear function of the number of methylene groups, *m*, in the imino acid molecule. Surprisingly, this relationship held true in a ternary solvent system F, as well as in the binary system G. This is indicative of minimal solvent demixing in these TLC systems. Extension of the straight lines in both directions allows the prediction of the R_F values of the lower and higher membered homologs of this series. Thus, the lowest cyclic homolog of proline, aziridine-2-carboxylic acid (*1a*), may be expected to have R_F values of 0.11 of 0.26 in solvent systems

* Related as the function of R_F as follows: $R_M = \log (1/R_F - 1)$.

F and G, respectively. The instability of this imino acid in the free form renders this prediction purely academic; nevertheless, the possibility remains that certain of its more stable derivatives might well relate similarly when compared to the corresponding derivatives of the higher members. The larger ring homologs, namely, those α-imino acids containing ten, eleven or twelve methylene groups are expected to be chemically more stable, and similar projections might give, for example, R_F values of 0.78 and 0.59 in solvents F and G for the as yet unreported azacyclododecane-2-carboxylic acid (1, $m = 10$).

It is noteworthy that 4,4-dimethylproline, a dialkylated proline which is isomeric with the 7-membered hexahydroazepine-2-carboxylic acid (*1e*) and which may be considered to contain four methylene groups in the molecule, did not fall on the straight line defined by the Martin relation. In fact, it behaved in solvent G as though it contained *six* methylene groups (however, *vide infra*).

TLC of the homologous DNP-, DNS- and PTH-α-imino acids

Table II lists the R_F values of the homologous series of DNP-, DNS- and PTH-α-imino acids in a number of TLC systems. DNP-DL-Pipecolic acid behaved quite anomalously in the two systems described, its R_F values deviating considerably from that expected for its position in the homologous series. DNP-L-Pipecolic acid behaved similarly. Sampling errors were eliminated as possible causes. This deviation

TABLE II

SEPARATION OF DNP-, DNS- AND PTH-DERIVATIVES OF HOMOLOGOUS CYCLIC α-IMINO ACIDS BY TLC

Silica Gel F_{254}, pre-coated plates for the DNP-, pre-coated silica gel without fluorescent indicator (E. Merck, A.G.) for the DNS-imino acids, and MN-Polyamid-DC 11 UV_{254} (Macherey, Nagel and Co., distributed by Brinkmann Instruments, Inc.), laboratory-coated plates, for the PTH-derivatives. The DNS-imino acids were applied as piperidinium salts, except for DNS-dimethylproline which was applied as the cyclohexylamine salt. Solvents: H = benzene–pyridine–acetic acid (40:10:1); I = 95% ethanol–benzene–H_2O (95:125:10); J = abs. ethanol–benzene–H_2O (95:125:10); K = heptane–*tert.*-butyl alcohol–acetic acid (20:5:8); L = carbon tetrachloride–benzyl alcohol–acetic acid (7:2:1); M = *n*-propyl alcohol–H_2O (1:2); N = abs. ethanol–H_2O (1:1); O = methanol–H_2O (2:1).

Number of methylene groups (m)	*Derivative of*	*R_F values in solvents*							
		DNP			*DNS*		*PTH*		
		H[a]	*I*[b]	*J*[c]	*K*[a]	*L*[a]	*M*[d]	*N*[d]	*O*[d]
2	L-Azetidine-2-carboxylic acid	0.11	0.36	0.34	0.17	0.37	0.40	0.48	0.55
3	L-Proline	0.21	0.41	0.41	0.24	0.48	0.39	0.46	0.54
3	DL-Proline	0.21	0.42	0.41	0.24	0.48	—	—	—
4	DL-Pipecolic acid	0.56	0.59	0.63	0.39	0.66	0.36	0.43	0.51
5	Hexahydroazepine-2-carboxylic acid	0.49	0.55	0.58	0.42	0.65	0.27	0.32	0.41
6	Octahydroazocine-2-carboxylic acid	0.59	0.63	0.66	0.48	0.71	0.23	0.26	0.34
7	Octahydroazonine-2-carboxylic acid	0.68	0.68	0.72	0.53	0.76	0.19	0.21	0.28
8	Decahydroazecine-2-carboxylic acid	0.74	0.73	0.78	0.56	0.78	0.15	0.15	0.21
9	Azacycloundecane-2-carboxylic acid	0.75	0.73	0.80	0.58	0.81	0.12	0.11	0.15
4	4,4-Dimethyl-DL-proline	0.36	0.49	0.52	0.37	0.59	0.25	0.30	0.41

[a] Average of 8 determinations. S.D. ≤ 0.02.
[b] Average of 2 determinations. S.D. ≤ 0.02.
[c] Average of 3 determinations. S.D. ≤ 0.02.
[d] Average of 5 determinations. S.D. ≤ 0.02.

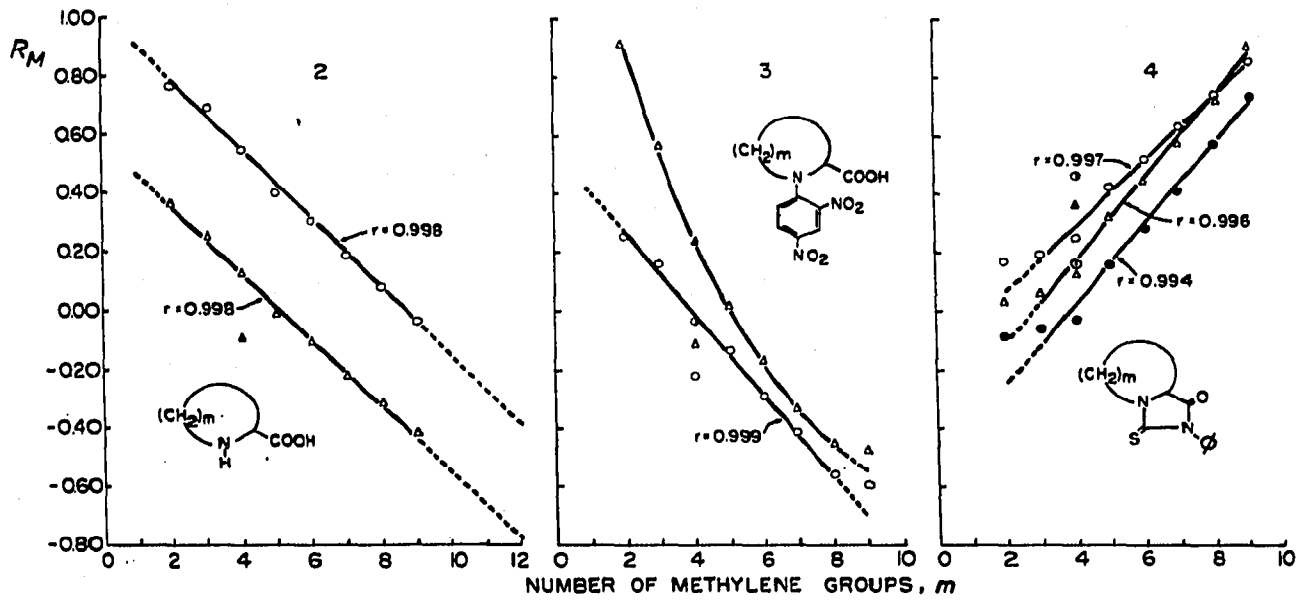


Fig. 2. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the α-imino acid molecule represented by the inset structure. O—O = Chromatographed in solvent F; △—△ = solvent G. ▲ = 4,4-Dimethyl-DL-proline in the latter system. The lines were drawn using the method of least squares; the correlation coefficients (r) are indicated. From the dashed extensions the theoretical R_M values for the yet unreported series of α-imino acids with 2, 10, 11, or 12 methylene groups can be found.

Fig. 3. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the homologous DNP-α-imino acids. △—△ = Solvent H; O—O = solvent J. ▲ and ◑ = DNP-derivative of 4,4-dimethyl-DL-proline in solvents H and J, respectively. Note that it is not possible to extend the line beyond $m = 9$ since linearity is not preserved here. The straight line was drawn using the method of least squares except that points at $m = 4$ and $m = 9$ (for DNP-pipecolic acid and DNP-azacycloundecane-2-carboxylic acid, respectively) were not included; the curved line was drawn through the points △ (except at $m = 4$, and $m = 9$).

Fig. 4. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the α-imino acid PTH-derivatives. O—O = solvent M; △—△ = solvent N; ●—● = solvent O. ◑, ▲ and ⊙ = PTH-derivative of 4,4-dimethyl-DL-proline in solvents M, N and O, respectively. The experimental points at $m = 2$ were not included in drawing the straight lines.

is reflected in the plot of R_M *vs.* number of methylene groups in the molecule (Fig. 3). Although a non-linear relation is observed for the homologous series in solvent H, a fairly linear relation obtains in solvent J. Both DNP-pipecolic acid ($m = 4$) and DNP-azacycloundecane-2-carboxylic acid ($m = 9$) fell outside their expected positions on the straight line. On the other hand, the points for DNP-dimethylproline coincided on the line (assuming four methylene groups) *in the expected positions for DNP-pipecolic acid* and might easily be mistaken for the latter. This appears to be purely fortuitous, as, it is recalled, dimethylproline itself as the free imino acid did not behave in this manner (see Fig. 2 above).

The PTH-derivatives of the homologous α-imino acids were not easily separable on TLC plates of silica gel, alumina or cellulose, and homologs differing by one methylene group overlapped each other in the variety of solvent systems tried. Efficient separations were achieved, however, on a polyamide matrix (Table II) (*cf.* WANG *et al.*[17]). The behavior of the PTH-derivatives on polyamide plates was reminiscent of reversed-

phase paper chromatography[18] in that the smaller molecular weight, less lipophylic members of the homologous series moved faster than the larger molecular weight highly lipophylic members. The Martin relation was again found to be valid (Fig. 4), and the correlation was quite satisfactory if the phenylthiohydantoin of azetidine-2-carboxylic acid ($m = 2$) was excluded.

ACKNOWLEDGEMENTS

This work was supported, in part, by Grant CA-06432, United States Public Health Service. The technical assistance of Miss LINDA MARTIN is gratefully acknowledged.

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