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INDUCTION OF CELL ARREST IN G2: STRUCTURAL SPECIFICITY OF TRIGONELLINE

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Abstract—Synthetic analogues of *N*-methyl nicotinic acid, trigonelline, were prepared to test the structural features necessary for the induction of cellular arrest in G2 in *Pisum sativum*. Analogues that (1) were regioisomers of trigonelline, (2) possessed different 1,3-substituents, and (3) contained additional substituents on the pyridine ring were tested for their ability to induce cell arrest in G2 and to antagonize trigonelline induced arrest in G2. Only *N*-methyl-3-quinoline-carboxylic acid and 1-methyl nicotinamide induced cell arrest in G2, and 1-methyl-4-pyridine carboxylic acid and 1-methyl-2-pyridine carboxylic acid were effective trigonelline antagonists. These data further support a specific role for trigonelline in the induction of cell arrest in G2.

INTRODUCTION

In mature root tissues and in root meristems under temporary carbohydrate deprivation, cells arrest in G1, in G2, or become polyploid. The relative proportions of cells arrested in these stages have been found to be constant, depending on the tissue [1, 2]. In *Pisum sativum* L., a substance, the G2 Factor, was identified in the cotyledons which was capable of altering the normal proportions of cell arrest [3]. Forty percent of the meristem cell population is preconditioned by this factor to arrest in G2 [4]. In mammals, proteinaceous substances that affect cell arrest or blockage in G1 or G2, called chalones, have been reported [5, 6]. These substances exhibit a cellular effect that is similar, but not identical to the G2 Factor. The G2 Factor was the first substance shown to alter the proportions of cell arrest in plant tissues and was characterized as the known alkaloid, trigonelline, **1** [7, 8].

Trigonelline biosynthesis in *P. sativum* diverts nicotinic acid from the production of pyridine adenine dinucleotides in the Priess–Handler metabolic pathway [9]. Nicotinic acid and nicotinamide are rapidly converted into trigonelline, whereas the reverse demethylation occurs only very slowly in *P. sativum* root cultures [10, 11]. None of the members of this pathway, including nicotinic acid and nicotinamide, are capable of altering the proportions of cell arrest in *P. sativum* [12]. This apparent structural specificity has prompted the present studies where several structural analogues of trigonelline have been investigated.

Previous reports [13] have shown that pyridine carboxylic acids can be efficiently methylated at nitrogen following esterification of the carboxylic acid. Slight variations of this procedure have allowed for the synthesis of the trigonelline analogues used in this study. The synthetic analogues have been selected to address the importance of three structural features of trigonelline: (1) the presence of the carboxyl group and the requirements for its regiochemical proximity to the quaternary nitrogen; (2) the requirements of the alkyl substituents on nitrogen; and (3) the necessity for an unsubstituted pyridine ring.

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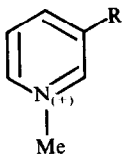
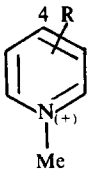
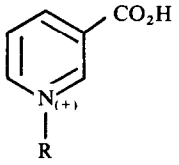
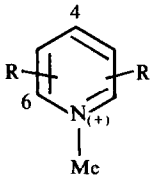
RESULTS

Table 1A contains four trigonelline analogues that explore the requirements for the carboxyl group. Replacement of this functional group by hydrogen, **2**, sulfate, **3**, or carboxy methylene, **4**, gave analogues which possessed no activity even at concentrations as high as 10^{-4} M. Analogues **3** and **4** were also assayed for their ability to block or antagonize trigonelline-induced cell arrest in G2, but neither analogue altered the effect of trigonelline. The amide, **5**, did promote cell arrest in G2. It is possible that this activity is due to the hydrolysis of **5** to trigonelline. While nicotinamide is known to be rapidly hydrolysed to nicotinic acid under similar conditions, [10–12] recent evidence suggests that **5** is not converted to trigonelline [Tramontano, W. A., unpublished].

Analogues **6** and **7** (Table 1B), whose carboxyl group regiochemistry differs from that of trigonelline, had no effect alone, but were markedly inhibitory to trigonelline-promoted cell arrest in G2. This antagonism is striking when compared to analogues with modifications at nitrogen (Table 1C). Neither **8** nor **9** were effective trigonelline antagonists. Although the *N*-ethyl derivative, **8**, does show some promotion of arrest in G2 at 10^{-4}, its antagonistic activity was not different from the *N*-benzyl compound, **9**.

Effects of further modifications of the pyridine ring are shown in Table 1D. Both the diacids, **11** and **12**, exhibited

Table 1 Proportions of cells arrested in G2 by the synthesized trigonelline analogues

	Analogue number	R	Proportion of cells arrested in G2			
			[Analogue]		10^{-6} M Trigonelline + [Analogue]	
			10^{-4} M	10^{-6} M	10^{-4} M	10^{-6} M
A						
	1	$CO_2\overline{H}$	0.62 ± 0.04	0 47 ± 0 02	—	—
	2	H	0.14 ± 0 01	0 22 ± 0.03	—	—
	3	SO_3H	0.15 ± 0.02	0.17 ± 0.02	—	0.36 ± 0 02
	4	CH_2CO_2H	0.16 ± 0.01	—	0.34 ± 0.04	—
	5	$CONH_2$	0 50 ± 0.02	0.36 ± 0 03	—	—
B						
	6	2-CO_2H	0.12 ± 0.01	0.16 ± 0.01	0.17 ± 0 01	0 39 ± 0 01
	7	4-CO_2H	0.14 ± 0.01	0.14 ± 0.01	0.20 ± 0.01	0 36 ± 0 01
C						
	8	CH_2CH_3	0.30 ± 0 01	0.17 ± 0.02	0.32 ± 0.02	—
	9	CH_2Ph	0.11 ± 0.02	—	0.30 ± 0.02	—
D						
	10	3-CO_2H, 5-CO_2H	—	0.15 ± 0.01	—	0.44 ± 0.02
	11	2-CO_2H, 5-CO_2H	0 30 ± 0 02	0.33 ± 0.02	—	0.30 ± 0.02
	12	3-CO_2H, 4-CO_2H	0 30 ± 0 01	0 37 ± 0.02	0.37 ± 0.02	0.37 ± 0.02
	13	3-CO_2H, benzo[e]	0 42 ± 0 02	0 17 ± 0 01	0.37 ± 0.01	0.38 ± 0.02
	14	Ricinine	(toxic)	0 16 ± 0.01	—	0.39 ± 0.02

Pisum roots were grown in medium containing sucrose and the indicated synthetic analogue and then transferred to fresh medium (without sucrose) to establish stationary phase Competition experiments were carried out by incubating roots in the presence of trigonelline (10^{-6} M) and the analogue (10^{-6} M or 10^{-4} M). Values of the proportions of cells arrested in G2 are reported as ± standard error of the mean from three experiments. Hyphens (—) indicate experiment not performed and the proportion of cell arrest with no addition is 0 14 ± 0.01

promotion of cell arrest in G2 that was independent of concentration and the symmetrical 3,5-diacid, **10**, had no effect. *N*-methyl-3-quinolinecarboxylic acid, **13**, an analogue with an aromatic system fused on the *e* face of the pyridine ring, promoted cell arrest in G2 at high concentrations (10^{-4} M) and did not antagonize promotion by trigonelline. The addition of this planar, nonpolar additional ring to trigonelline gave one of the most effective trigonelline mimics that has been tested.

DISCUSSION

Previous results from our laboratories [10–12] have shown that of all the Priess–Handler intermediates in the

biosynthesis of NAD, only trigonelline is capable of promoting cellular arrest in G2 in developing *P. sativum* roots. While higher concentrations of many of these intermediates will induce preferential cell arrest in G2, biosynthetic studies with nicotinic acid and nicotinamide, direct precursors of trigonelline, have shown that this effect can be explained by their biosynthetic conversion into trigonelline.

These same biosynthetic studies demonstrated that trigonelline is not metabolized further during the time period required for its effect. These data support the contention that trigonelline is serving as a signal for inducing preferential cell arrest in G2 [14], and led to the investigation of the synthetic analogues reported in this paper.

Both the carboxyl group and the alkylated quaternary nitrogen [12] of trigonelline are required for activity. Movement of the position of the carboxyl group on the pyridine ring (Table 1B) gave analogues that could not mimic the effect of trigonelline on cell arrest, but at high concentrations these derivatives did antagonize trigonelline action.

The placement of additional carboxyl groups on the pyridine ring gave results that are not readily explained. There is little difference in the levels of cells in G2 with or without added trigonelline and irrespective of the concentration of the analogues. This apparent lack of structural and concentration dependence does not appear to be a non-specific toxicity affect. The toxic alkaloid, ricinine, **14**, which is a further metabolite of trigonelline in some tissues [14], does not alter the percentage of cells in G2.

Increasing the steric bulk of the substituent on nitrogen markedly alters the ability of the analogue to promote arrest in G2. Replacement of the *N*-methyl of trigonelline with an ethyl group, **8**, gives an analogue possessing only weak activity. The steric requirement of the pyridine ring is not as critical. The quinoline derivative, **13**, has reduced activity, but at high concentrations the proportions of cells arrested in G2 approach the levels induced by trigonelline.

Collectively, these data demonstrate an absolute requirement for the carboxyl group and the *N*-methyl group [12] of trigonelline. Variations in the structure or the regiochemical position of the carboxyl group lead to analogues that are completely devoid of activity. Slight structural changes in the *N*-methyl functionality markedly reduce the activity. While it is not clear what precise molecular interactions these analogues mediate in the alteration of the trigonelline-induced preferential cell arrest, these data do support a structural specificity associated with trigonelline action. We have previously observed that, in culture, *P. sativum* roots accumulate relatively high concentrations of trigonelline [4], but that G2 arrest is correlated with the nutrient medium concentration rather than the levels in the tissue. Although the reasons for this high concentration of trigonelline in the tissue are not understood, the observation is consistent with trigonelline serving as an intercellular signal for the induction of cell arrest in G2.

EXPERIMENTAL

Seeds of *Pisum sativum* were surface sterilized with undiluted Chlorox(R) that contains 5.25% Na hypochlorite, stirred frequently for 10 min, washed with sterile H_2O to remove bleach, and germinated in sterile vermiculate.

^{1}H NMR spectra were recorded on a Varian EM 390 or a NTC 360. MPs are uncorr. 1-Methylnicotinamide iodide was obtained from Sigma.

G2 Factor assay. The general culture techniques to determine the promotion of cell arrest in G2 have been described [17]. Excised root tips 15 mm long from 3-day-old seedlings were either cultured in medium without sucrose to establish a stationary phase meristem or placed in medium with sucrose for several days before carbohydrate deprivation and establishment of stationary phase The analogues with and without trigonelline (10^{-6} M) were placed in medium containing sucrose and the excised root tips for three days. The root tips were then transferred to basic media without the analogues or sucrose for the establishment of stationary phase. The data in Table 1 were obtained within the terminal 2 mm of the meristem.

DNA measurements. Measurements by relative DNA per nucleus of Feulgen-stained nuclei were obtained by microfluorimetry [17, 18]. The incident light passed a 545 nm interference filter (9 nm band width) and was focused with a dark field condenser onto individual Feulgen-stained nuclei. Feulgen stain fluorescence of light greater than 580 nm was recorded by a red-sensitive photomultiplier tube (Microspectrum Analyzer, Model 138119, Farrand Corp., Valhall, N.Y) The relative amout of DNA per nucleus was normalized with readings of one-half telophase and prophase mitotic figures taken to 2C and 4C values, respectively. Cells with or near a 2C DNA value were assumed to be in G1 while those with a 4C DNA value were assumed to be in G2.

N-Methyl nicotinic acid (trigonelline). Prepared essentially by the methods of ref [13]. Nicotinic acid was esterified in dry MeOH with 1 5 equivalents of H_2SO_4 at reflux for 24 hr. The mixture was cooled, neutralized with $NaHCO_3$ (dry), and dried *in vacuo*. Extraction of the residue with Et_2O gave quantitative recovery of methyl nicotinate which was dried and methylated with iodomethane (1.1 eq in C_6H_6, reflux 3 hr) Partitioning of this mixture with H_2O gave an aq. soln of 1-methyl-3-carbomethoxy pyridinium iodide which on passage through Dowex 1 (OH^- form) X8 and concn gave a semi-solid paste. Crystallization from EtOH gave trigonelline (mp 230–233°) in 65% overall yield.

Homanine hydrichloride Obtained from Aldrich and found pure by TLC ($CHCl_3$–MeOH, 95:5).

N-Methyl isonicotinate. Prepared by the same procedure as trigonelline using isonicotinic acid. Comparable yields ^{1}H NMR (90 MHz, D_2O). δ8 40 (2H, *d*, H-2, H-6), 7.93 (2H, *d*, H-3, H-5) 4.33 (3H, *s*, Me).

N-Ethyl nicotinic acid. Prepared by the same procedure as trigonelline using iodoethane. The ethylation gave a fair yield and was purified by passage through Dowex ^{1}H NMR (D_2O, 90 MHz) δ9.12 (*br s*, H-2), 8.76 (H-4, *d*, J = 7 2 Hz), 8 02 (H-5, *t*, J = 7 2 Hz), 8 83 (H-6, *d*, J = 7 2 Hz), 4.65 (CH_2, *s*, J = 6 5 Hz), 1 61 (Me, *t*, J = 6.5 Hz).

N-Benzyl nicotinic acid Prepared by the same procedure as trigonelline using benzyl bromide. Benzylation produced moderate yields and the product eluted cleanly from Dowex. ^{1}H NMR (D_2O, 90 MHz): δ9.23 (*br s*, H-2), 8.83 (H-6, *d*, J = 6.0 Hz), 7.70 (H-4, *d*, J = 8.0 Hz), 7.98 (H-5, *dd*, J = 6.0, 8.0 Hz), 7.36 (5H, *s*, C_5H_5), 5.73 (2H, *s*, CH_2)

N-Methyl-3,5-dicarboxypyridine The pyridine dicarboxylic acid was esterified in dry MeOH with 1 5 eq. H_2SO_4 (mp 81.5–82.5°, lit. [8] 80.5–82 5°). The diester was methylated with excess dimethylsulphate in refluxing C_6H_6 for 6 hr The resulting sulphate salt was extracted into H_2O and passed through Dowex 1 (OH^- form) X8 and the product eluted with H_2O. The resulting betaine could be chromatographed on SiO_2 ($CHCl_3$–MeOH–H_2O, 65 35:10) and further purified on Sephadex G-15 in H_2O.

^{1}H NMR (D_2O, 90 MHz): δ9.27 (2H, *d*, H-2, H-6, J = 2.0 Hz), 8.76 (*t*, H-4, J = 2.0 Hz), 4.36 (3H, *s*, Me).

N-*Methyl*-3,4-*dicarboxypyridine*. The dicarboxylic acid was esterified and methylated by the methods used for the 3,5-diacid. Overall yields were moderate, and Sephadex G-15 allowed for final purification. ^{1}H NMR (D_2O, 90 MHz): δ9.11 (*br s*, H-2) 7.90 (*br d*, H-5, J = 7.0 Hz), 8.75 (*br d*, H-6, J = 7.0 Hz), 4.21 (3H, *s*, Me).

N-*Methyl*-2,5-*dicarboxypyridine*. The dicarboxylic acid was esterified and methylated by the methods used for the 3,5-diacid. Overall yields were moderate, and Sephadex G-15 or G-10 allowed for final purification. ^{1}H NMR (D_2O, 90 MHz): δ9.16 (*d*, H-6, J = 2.0 Hz) 8.86 (*dd*, H-4, J = 8.0 and 2.0 Hz); 8.02 (*d*, H-3, J = 8.0 Hz), 4.31 (3H, *s*, Me).

N-*methyl*-3-*pyridylacetic acid*. Esterification of pyridylacetic acid in MeOH–H_2SO_4 and nitrogen quaternarization with methyl iodide in refluxing C_6H_6 proceeded smoothly. Passage of the salt through Dowex 1 (OH^- form) produced the betaine in good yield and final purification was accomplished by Sephadex G-15 chromatography. ^{1}H NMR (D_2O, 90 MHz): δ8.56 (*br s*, H-2), 8.27 (*d*, H-4, J = 8.0 Hz), 7.84 (*dd*, H-5, J = 6.0 and 8.0 Hz), 8.52 (*d*, H-6, J = 6.0 Hz), 3 66 (2H, *s*, CH_2) 4.26 (3H, *s*, Me).

N-*Methyl*-3-*quinolinecarboxylic acid*. 3-Quinolinecarboxylic acid was esterified and methylated by procedures analogous to those used for trigonelline. The product was obtained in moderate to good yield and final purification was accomplished by Sephadex G-15 chromatography ^{1}H NMR (acetone-d_6, 360 MHz): δ8.26 (*s*, H-2), 8.23 (*s*, H-4) 7 28 (*d*, H-8, J = 8.0 Hz), 7.26 (*d*, H-5, J = 8 0 Hz), 7.02 (*t*, H-6, J = 8.0 Hz), 6.70 (*t*, H-7, J = 8.0 Hz)

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