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A

**ANTIOXIDANT DITERPENOIDS FROM CALLUS
AND REGENERATED PLANTS OF
*Rosmarinus officinalis***

By

PATRICIA HARRIGAN NADOSY

**A dissertation submitted to the Graduate Faculty of Biology in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York**

2002

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

**ANTIOXIDANT DITERPENOIDS FROM CALLUS
AND REGENERATED PLANTS OF
*Rosmarinus officinalis***

by

Patricia Harrigan Nadosy

Advisor- Professor Dominick V. Basile

The phenolic diterpenoids, carnosic acid and carnosol, are powerful antioxidant and antimutagenic compounds in animal systems. Though the levels of these natural products are high in cultivated and wild rosemary, the yields are inconsistent and highly variable. This dissertation research was designed to determine if these phenolic diterpenoids are produced in undifferentiated cells and regenerated shoots of rosemary. The research was conducted in two phases. In the first phase, solid and liquid media formulations and tissue culture systems were devised for the production of callus and regenerated plants of rosemary. In the second phase, these cultured tissues were extracted and analyzed for their phenolic diterpenoid composition. The diterpenoid components and yields of extracts from rosemary tissues grown *in vitro* and *in terra* were compared.

The addition of high levels of BAP to MS basal media with IAA and kinetin and the maintenance of cultures at elevated temperature and high illumination resulted in the relatively rapid regeneration of rosemary plants with one long initial passage and no

required changes in media formulation. Two different media formulations were found to produce healthy green or white callus. Elevated temperatures and light levels enhanced the growth of regenerated shoots, while lower light and temperature levels ensured the maintenance of callus.

Four phenolic diterpenoids, carnosic acid, carnosol, rosmanol, and 12-methoxy carnosic acid, were extracted from shoots and flowers of soil grown rosemary as well as from rosemary callus and regenerated shoots grown in tissue culture. The yields of these compounds were substantially lower in the extracts of the plant material grown *in vitro* than in the extracts of cultivated rosemary shoots and flowers. Extracts containing detectable levels of these compounds were obtained by devising improved isolation procedures, including a protocol that resulted in carnosic acid being the major component of the extracts. The ability to obtain relatively high proportions of carnosic acid facilitated the crystallization of this powerful antioxidant compound.

To
my family, who inspired and encouraged me
and gave me the strength and the time to pursue my dream.

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I would like to thank Lorna Price, not only for granting me permission to reproduce the Plan of the St. Gall Medicinal Herb Garden, but also for providing a scan from the original document and in record time. I am also grateful to Robert Dittmer for his technical expertise.

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I will always be indebted to Professor Philip Ammirato of Barnard College who started me on the path to botanical research and who is always already to share ideas and discoveries. Although the asparagine monohydrate research is not part of this dissertation, I would like to thank Professors Leslie Lessinger and Christian Rojas of Barnard College for helping me to solve this mystery by analyzing the crystals.

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CHAPTER ONE

Introduction

Centuries before the term antioxidant was used in general parlance and long before herbs were studied for their antioxidant properties, humans used *Rosmarinus officinalis* Linn. to promote good health. Rosemary (**Figure 1.1**) is an evergreen subshrub, indigenous to the Mediterranean, with dark green, revolute, needle-like leaves on ash colored bark and typically purple calyxes. The genus *Rosmarinus* is in the subfamily Nepetoideae of the Lamiaceae family. The number of species of rosemary is cited variously as four [Turrill 1920],¹ twelve [Browse],² or two [Arnold *et al.* 1997].³ A recent scan of the catalogues of East coast purveyors of herb plants suggests that *Rosmarinus officinalis* is effectively the only species available in the United States, though there are well over forty cultivars, including Albus, Arp, Collingwood Ingram, Gorizia, Hill Hardy, Ingram, Lockwood de Forest, Miss Jessup, Prostratus, Salem, Tuscan Blue. Scientific literature of the last twenty years reveals three species available for research in southern Europe: *Rosmarinus officinalis* Linn., *Rosmarinus eriocalyx* Jordan & Fourr. [Tucker and Maciarello 1986; Arnold *et al.* 1997], and *Rosmarinus tomentosus* Huber-Morath & Maire [Rosua and Garcia-Granados 1987; Navarro 1993]. The latter two species are endemic to Algeria, Morocco, and southern Spain, but *R. tomentosus* is currently protected in Northern

¹ *R. officinalis*, *R. laxiflorus*, *R. lavandulaceus*, *R. Tournefortii*.

² M. B. Browse referred back to Turrill who presented a revision of the previous ranking of 12 species.

³ *R. officinalis* and *R. eriocalyx*. Arnold *et al.* considered *R. laxiflorus* to be synonymous with *R. officinalis*.

The name rosemary is from the Latin *rosmarinus*⁴ “dew of the sea,” but rosemary was known in ancient Greece and in Egypt during Roman times [Barakat and Baum 1992]. Dioscorides, a Greek physician who wrote *De Materia Medica*, the first extant Western herbal, referred to rosemary as *Libanotis*,⁵ and attributed it with “. . .a warming facultie. . .”[Gunther 1933]. In the late twentieth century, Aqel found that the volatile oil of rosemary inhibited the contraction of rabbit aortic rings and rabbit and guinea pig tracheal smooth muscle [Aqel 1991, 1992]. He attributed this relaxant effect to interference with calcium influx and intracellular calcium release.

The complete story of rosemary’s use in Egypt is still emerging. In 1581, a Venetian medical doctor, Prosper Alpin, traveled to Egypt to study diseases and remedies, including the use of local plants. In examining one tomb, Alpin [1979] found branches of rosemary that were “. . .as green and fresh as if they had been collected and placed there the same day.”⁶ His “discovery” has been dismissed by several researchers, and Bonnet [1905], in particular, believed that Alpin was duped, “il avait été victime d’une supercherie.” Bonnet himself reported finding branches of rosemary in a terra cotta jar in the tomb of a young woman in Antinoé, along with marjoram, rose petals and pieces of a lichen [Bonnet 1905]. More recently, during examinations of fourteen tombs in 1982 and another four in 1990 near the Kharga oasis, Barakat and Baum [1992] identified and collected more than twelve

⁴ *Ros* - dew and *marinus* - sea.

⁵ Pliny used the word *Libanotis*, but remarked that “Some people call it by another name, *rosmarinum*.” Rackam, V, XVIII.

⁶ “. . .aussi vertes et fraîches que si elles avaient été cueillies et placées là le jour même.”[Alpin 1979]

rosemary branches (with and without leaves) and one inflorescence as parts of bouquets. The researchers remarked on the diversity of cultivated plant material present in the tombs; the discovery of rosemary was particularly surprising since it was not native to Egypt. Prosper Alpin's report has been regarded as suspect due to his description of the freshness of the plant material, and since his report has been widely disseminated, many researchers have concluded that we will never know if the Egyptians used rosemary. However, combining the discoveries by Bonnet [1905] and by Barakat and Baum [1992], which up to now has not been done, certainly does confirm that the early Egyptians cultivated and used rosemary.

Rosemary's reputation as the herb of remembrance may, in fact, originate in the Egyptian custom of placing of these branches in the tombs of those who wanted to be remembered forever. Another possible source of rosemary's renown as the herb of remembrance originates in the legend that in ancient Greece students wore garlands woven of the herb's shoots during examinations to enhance their recall. Certainly, weaving garlands of rosemary was a practice in early Roman times for aesthetic as well as intellectual purposes:

She comb'd and set in curls, her auburn hair
 Of roses, violets, and lillies mix'd,
 And sprigs of flowing rosemary betwixt,
 She form'd the chaplet, that adorn'd her front.

Ovid. *Metamorphoses*, Book XII, lines 545-548 [Ovid]

Nicholas Culpeper, a seventeenth century English herbalist [Culpeper 1653], also believed in rosemary's cognitive powers, "It helps a weak memory, and quickens the senses." The view of rosemary as a mental stimulant is also revealed by Ophelia's plaint:

"There's rosemary, that's for remembrance;
prayer, love, remember . . ."

Hamlet, Act IV, Scene VI, lines 175-176

[Rowse, 1978]

Recently, researchers at the University of Miami School of Medicine observed that students, after inhaling rosemary oil, felt more alert and relaxed, and performed math calculations more quickly than did the control group [Diego *et al.* 1998].

Modern science has also confirmed the ancient Greek medical practice of including rosemary in a diuretic mixture, as recorded by Aretaeus, a contemporary of Hippocrates. According to Jerry Stannard, Aretaeus recommended renal calculi therapy starting with a bath containing blended herbs (rosemary, dill, and marjoram), followed by a drink with the same herbs [Stannard and Kay 1999]. Haloui and his colleagues reported, in the year 2000, that 8% aqueous extracts of rosemary, prepared in the traditional Moroccan way, administered daily to adult male Wistar rats, enhanced diuresis from the fifth day [Haloui *et al.* 2000].

Rosemary's documented use as a medicinal remedy and seasoning dates from the Middle Ages. Some hypothesize that because of rosemary's role in sacred ceremonies, early writers did not describe its use for such mundane purposes as flavoring foods. The first written documentation that rosemary was used on a daily

basis was found in the plan of the herb garden of the monastery of St. Gall in Switzerland. The plan of this garden (next to the infirmary) which dates from the time of Charlemagne,⁷ shows rosemary as one of the sixteen plants (Figure 1.2).

Shakespeare praises rosemary's preservative properties in *The Winter's Tale*, a play replete with pastoral references, when Perdita offers sarcastically,

“ . . . Reverend sirs,
For you there's rosemary and rue; these keep
Seeming and savour all the winter long:”

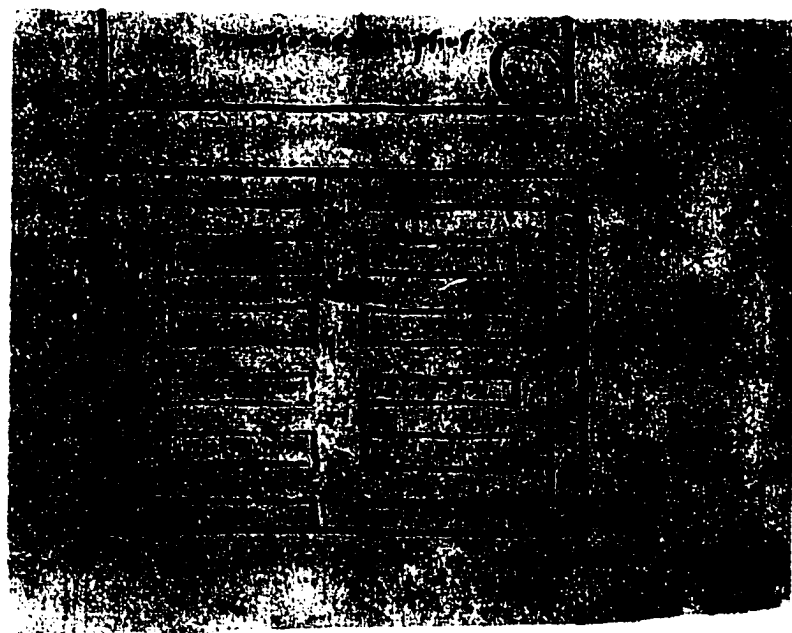
The Winter's Tale, Act IV, Scene IV, lines 74-76

[Rowse 1978]

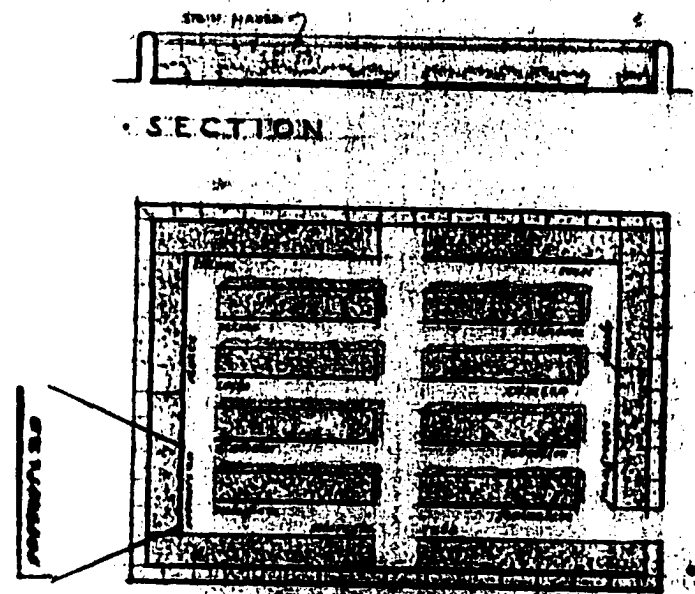
The recognition of rosemary's antioxidant properties may have originated in ancient Greece where legend has it that fishermen wrapped their fresh catch in rosemary shoots to prevent spoiling though there is no written record of this. Journals of nutrition today abound with papers demonstrating the power of rosemary extract to improve shelf life of foods [Bradford *et al.* 1994], and to prevent the oxidation of food lipids [Frankel *et al.* 1996; Martínez-Tomé *et al.* 2001].

Rosemary has also been useful to humans for its now well documented anti-bacterial and anti-fungal effects [Farghaly *et al.* 1989; Panizzi *et al.* 1993]. For this reason and others, it is one of the few herbs that have been used for centuries at the most pivotal ceremonies in life, weddings, and funerals. Rosemary's antiseptic

⁷ The plan was “copied between 820-830 A.D. from a lost original.” [Price, 1982].



PLAN OF ST. GALL. MEDICINAL HERB GARDEN



MEDICINAL HERB GARDEN

AUTHORS' INTERPRETATION

SCALE: $\frac{1}{4}$ INCH EQUALS 1 FOOT (1:192)

Figure 1.2 Plan of the medicinal herb garden at the monastery of St. Gall, *circa* 800 A.D., with an interpretation by Price [Price 1982]. Enlargement of the word *rosmarino* has been added.

Note: Born rotated the scheme clockwise 180° so the plant names would 'read.' The scribe, for purposes of his own, turned the work as he filled in the plant names (personal communication Lorna Price, April 2002).

Reproduced with the permission of the estate of Ernest Born.

properties recommended it for funerals; Culpeper suggested burning rosemary “ . . . to expell the contagion of the pestilence.” Its reputation as the herb that symbolizes faithfulness made it suitable to weddings. Rosemary’s place at these ceremonies was well established by 1648,

The Rosemarie branch

Grow for two ends, it matters not at all,
Be’t for my Bridall or my Buriall.

Robert Herrick *Hesperides*
From [Patrick 1963]

Early herbals [Culpeper 1653; Gerard 1653] also listed numerous medicinal benefits bestowed by rosemary tisanes, infusions, poultices, and rosemary oil. For example, Gerard [1653] cited the Arabians for using rosemary to “openeth the floppings of the liuer . . .” Hoefler *et al.* [1987] validated the cholaretic and hepatoprotective properties of ethanol extracts of rosemary in rats. The effectiveness of many of these folk remedies has been confirmed by modern scientific research as reviewed recently by Al-Sereiti *et al.* [1999]. The medicinal uses of rosemary are not limited to Europe. The Caribs of Guatemala use a decoction of the stem and leaf for stomach and menstrual pains and coughs [Giron *et al.* 1991]. In Baja California Sur (Mexico), *Lepechinia hastata*, which contains carnosol, was reported to cure uterine infections [Dimayuga *et al.* 1991]. For the most part, the folkloric and ethnobotanical reports concern aqueous and alcoholic extracts of rosemary so the effects rely on the

volatile compounds, such as the monoterpenoids. One famous legend of such an extract is the so-called Hungary water, which is said to be an infusion of rosemary leaves in alcohol. This tonic reportedly cured the aging Queen of Hungary from rheumatism or gout [Domokos *et al.* 1995].

The uses of rosemary for human health are numerous and varied, but today the antioxidant effects are of primary interest. These antioxidant properties convey positive effects on cardiovascular fitness, youthfulness, food preservation, and neutralization of free radical damage. Carnosic acid (**Figure 1.3**), a phenolic abietane diterpenoid with the ferruginol skeleton, is the compound primarily responsible for the antioxidant activity of rosemary extracts [Schwarz and Ternes 1992b; Aeschbach and Philippossian 1993; Richeimer *et al.* 1999]. In 1965, Wenkert and his colleagues were the first to identify carnosic acid in a plant extract (*Rosmarinus officinalis*), but it was not until 1993 that carnosic acid was isolated from plant material [Paris *et al.* 1993].⁸ This diterpenoid has subsequently been isolated from *Rosmarinus officinalis* by others [Aeschbach and Philippossian 1993; Paris *et al.* 1993; Richeimer *et al.* 1996], from six species of *Salvia*: *S. officinalis* [Narayanan and Linde 1965], *S. apiana* [Dentali and Hoffman 1990], *S. canariensis* [Gonzalez *et al.* 1987], *S. columbariae* [Luis *et al.* 1994], *S. mellifera* [Luis *et al.* 1992], *S. willeana* [de la Torre *et al.* 1990] and from *Lepechinia meyeri* and *Lepechinia hastata* [Bruno *et al.* 1991].

⁸ Though this group refers to the compound as “carnosolic acid,” they state that the spectral data matches that of carnosic acid reported by Wenkert *et al.* [1965].

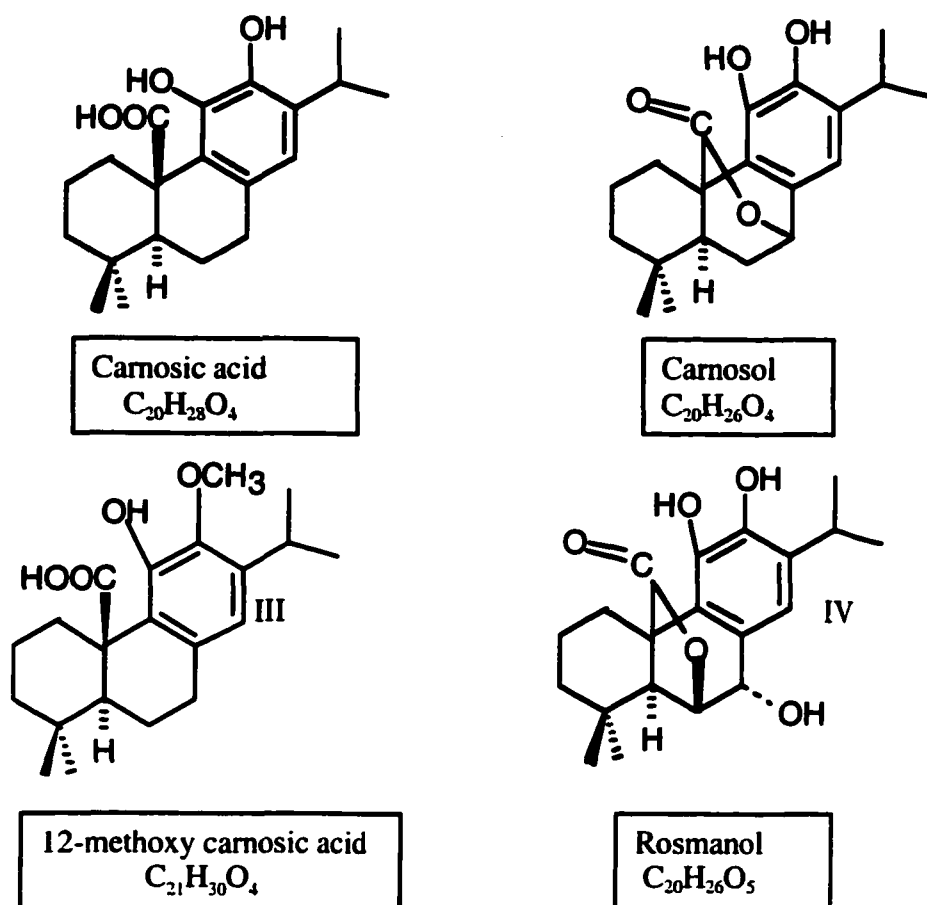


Figure 1.3 Structures and molecular formulae of four major phenolic diterpenoids from rosemary, carnosic acid (I), carnosol (II), 12-methoxy carnosic acid (III), and rosmanol (IV).

Other phenolic diterpenoids isolated from rosemary include: carnosol, rosmanol, 12-methoxy carnosic acid (**Figure 1.3**), rosmadial (a seco-diterpene lactone), rosmariquinone, epirosmanol, isorosmanol, (**Figure 1.4**), epiisosrosmanol, 7-ethoxyrosmanol, 7-methoxyrosmanol, 14-methoxy-7-*O*-methylrosmanol, 14-hydroxy-7-*O*-methylrosmanol, 7-*O*-methylrosmanol.

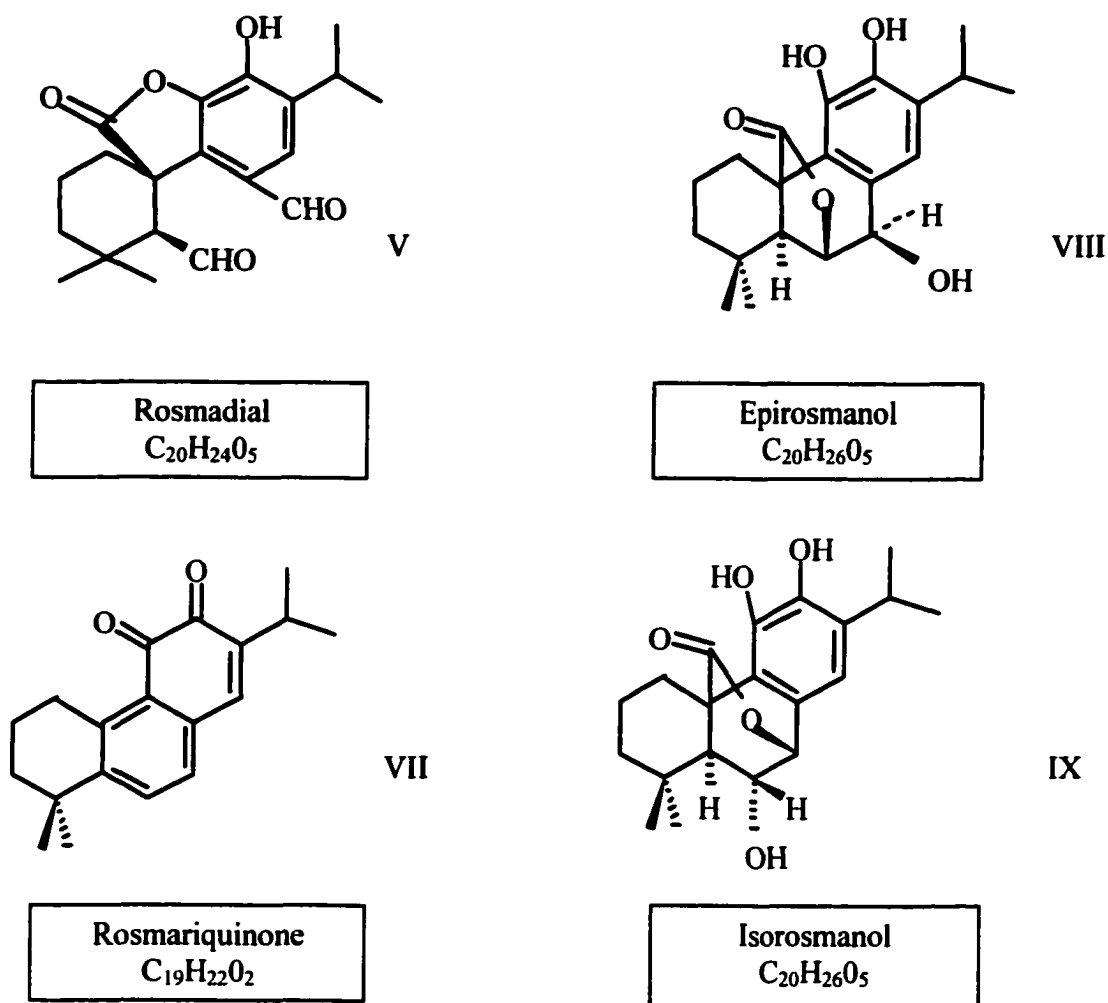


Figure 1.4 Structures and molecular formulae of four minor diterpenoids from rosemary, rosmadial (V), rosmariquinone (VII), epirosmanol (VIII), and isorosmanol (IX).

Carnosol, which possesses about one tenth the antioxidant strength of carnosic acid, was actually discovered first. In 1942, White isolated and purified carnosol from *Salvia carnosia*, a plant used for medicinal purposes by Native Americans in Minnesota. Carnosol has subsequently been isolated from all the species containing carnosic acid as mentioned above, plus *Isodon grandiflora* var. *Atuntzensis* C.Y. Wu, a member of the Labiatae with wide distribution in the Yunnan province of China

[Shun-Hua *et al.* 1993]. The researchers were surprised by this discovery since the genus *Isodon* tends to contain *ent* kaurane-type diterpenoids, not abietane type diterpenoids that are more characteristic of *Rosmarinus* and *Salvia*.

The antioxidant activity of carnosic acid is primarily a result of its ability to donate hydrogen atoms, from the ortho hydroxyl groups at positions 11 and 12, to free radical species such as singlet oxygen. Inatani and his colleagues proposed, based on structure-activity relationships, that the hydroxyl at position 11 was the more important [Inatani *et al.* 1982]. Masuda recently confirmed that the hydroxyl group at position 11 starts the oxidation process by donating a hydrogen to a radical species, such as a lipid peroxy radical, and forms a carnosate radical [Masuda *et al.* 2001]. 12-methoxy carnosic acid, with a methyl group in place of the hydroxyl at position 12, has no antioxidant properties [Richheimer *et al.* 1999].

Antioxidant effects have been reported for carnosol [Singletary and Nelshoppen 1991], rosmanol [Inatani *et al.* 1983], epirosmanol and isorosmanol [Nakatani and Inatani 1984], rosmaridiphenol [Houlihan *et al.* 1984], and rosmariquinone [Houlihan *et al.* 1985]. In studying commercial antioxidant mixtures for antiviral powers, Aruoma *et al.* [1996] found that purified carnosol inhibited HIV infection at a low, non-cytotoxic concentration. Paris' group also found that carnosic acid had an inhibitory effect on HIV-1 protease activity and on HIV-1 virus replication [Paris *et al.* 1993]. Aeschbach and Philippossian also reported antiviral effects from carnosic acid [Aeschbach and Philippossian 1993].

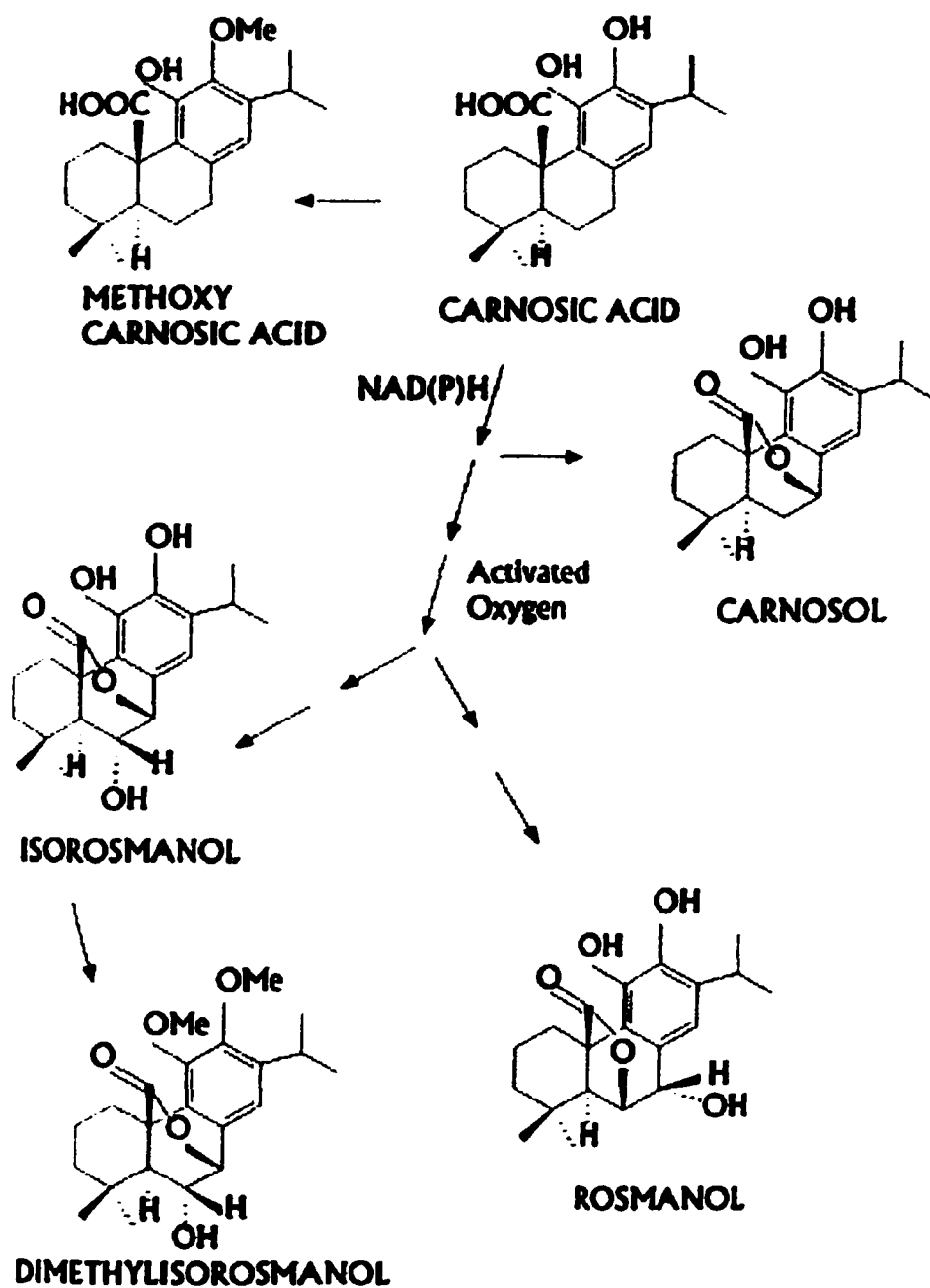
Aeschbach and his colleagues identified carnosic acid as the primary natural product of rosemary: "Carnosic acid is therefore the only phenolic diterpene present

in the native state in rosemary and sage and, accordingly, has the sole right to be called a natural product.” [Aeschbach and Philipposian 1993]. Richheimer’s team considered the number of natural phenolic constituents to be two, by including 12-methoxy carnosic acid: “Hence, CA and 12-MCA are the only phenolic diterpenes that can lay undisputed claim to being natural constituents of rosemary.” [Richheimer *et al.* 1996]. Their analysis was based on tests of the oxidation power of more than eight diterpenoids isolated from dried and fresh rosemary leaves and commercial extracts. Carnosic acid and 12-methoxy carnosic acid were present in every extract.

Most of the phenolic diterpenoids in rosemary have been studied and compared with regard to their antioxidant power in different systems. Though the data are somewhat difficult to reconcile given the application of different parameters, the overwhelming conclusion is that carnosic acid is the most powerful antioxidant with carnosol a distant second. Several recent reports validate the conclusions of earlier researchers. For example, Richheimer *et al.* [1999] confirmed the discoveries of Chen’s group and Cuvelier’s group that carnosic acid was more powerful than BHT and BHA as measured using the Rancimat method [Cuvelier *et al.* 1990; Chen *et al.* 1992]. Richheimer and his colleagues suggested, in 1999, that the power of the commercial extracts was actually partly attributable to the fact that the compounds, which derive from carnosic acid during the oxidation process, are themselves antioxidants. Specifically, carnosol and rosmanol which have been identified as potent antioxidants [Nakatani and Inatani 1981; Haraguchi *et al.* 1995] are stable at high temperatures, as is carnosic acid, and, therefore, can contribute to the efficacy of

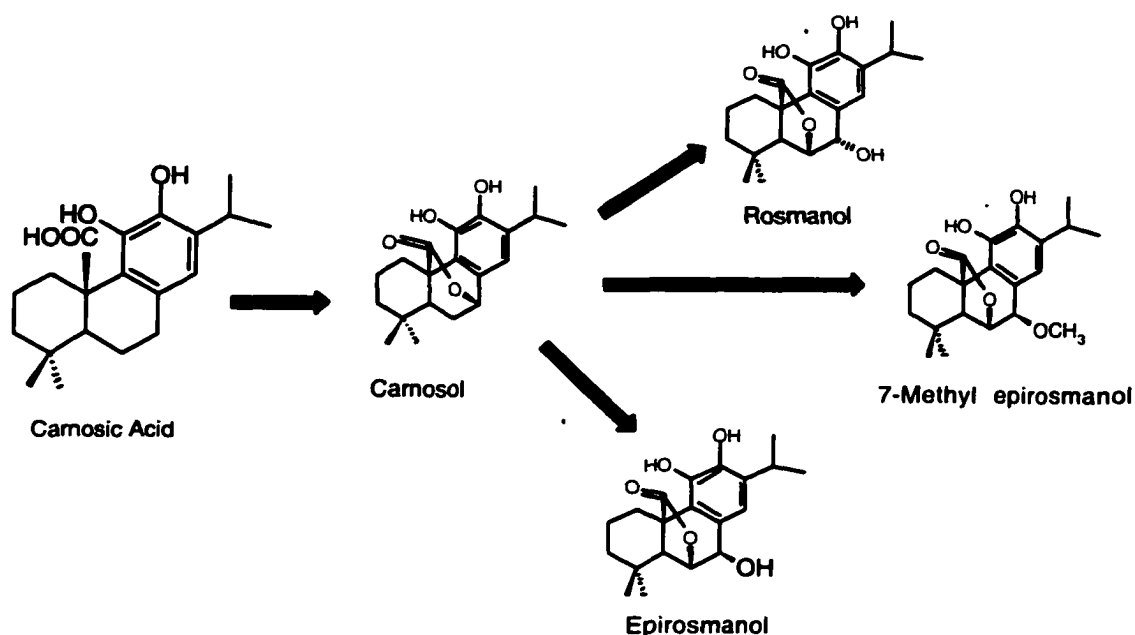
these extracts in a lipid system. Nakatani and Inatani found [1981] that the antioxidative activity of rosmanol to be four times that of BHA and BHT.

Carnosic acid's ready oxidation into derivative compounds may provide protection to the plant from damage due to radical species such as singlet oxygen, according to recent theories proposed by Munné-Bosch's group. As they recently found, carnosic acid oxidizes *in vivo* into the highly oxidized rosmanol, isorosmanol and dimethyl isorosmanol (**Scheme 1.1**) and is not regenerated [Munné-Bosch and Alegre 2001]. This ready oxidation also accounts for carnosic acid's reputation as a food preservative, as an antioxidant, and as antibacterial and antifungal agent. It also contributes to difficulties in the laboratory in extracting carnosic acid alone, without derivatives or as some researchers believe "artifacts."



Scheme 1.1 Degradation of carnosic acid in high light, high temperature, and low water levels into more highly oxidized compounds, isorosmanol, rosmanol, and dimethylisorosmanol. From Munné-Bosch *et al.* 1999a.

Carnosic acid may be converted to more highly oxidized derivatives during the extraction process. In 1992, Schwarz and Ternes [1992b] confirmed earlier work by Wenkert's group [Wenkert *et al.* 1965; Brieskorn and Domling 1969] that carnosic acid in methanol degrades into carnosol, which in turn decomposes into the more highly oxidized γ and δ lactones, rosmanol, epirosmanol and 7-methyl epirosmanol:



Scheme 1.2 Degradation of carnosic acid into four phenolic diterpene “artifacts” with γ - and δ -lactone structures: carnosol, rosmanol, 7-methyl epirosmanol, and epirosmanol. From Schwarz and Ternes 1992b.

Schwarz and Ternes [1992b] found that the amount of carnosol increased as the amount of carnosic acid decreased. It is now known, based on recent research by Munné-Bosch's team that once degraded *in vivo*, carnosic acid, unlike alpha-

tocopherol and other antioxidants, is not regenerated [Munné-Bosch and Alegre 2001].

Most carnosic acid derivative compounds are themselves antioxidants.

Richheimer's group postulated that carnosic acid containing mixtures may have "a longer antioxidant life span" than other antioxidants because of the chain of compounds initiated by the degradation of carnosic acid [Richheimer *et al.* 1996].

Rosemary's ascent in the hierarchy of antioxidants useful to humans in modern times was initiated by a 1952 analysis by Chipault and his colleagues of the antioxidant properties of thirty-two spices tested on prime steam lard using the active oxygen method. Rosemary and sage contained the highest percentage of compounds effective against the oxidation of foodstuffs [Chipault *et al.* 1952]. This information was cited in a report of the development of the first laboratory extraction of antioxidant compounds from rosemary in 1955. Rac and his colleagues, concerned about Yugoslavia's war-time dependence on imported products for food preservation and the associated costs, developed an effective extraction process for rosemary [Rac and Ostric 1955]. Since that time, extracts of rosemary have become widely used as preservatives in the food industry [Tsimidou and Boskou 1994; Hopia *et al.* 1996]. Multinational food processors market complex mixtures containing rosemary antioxidants, as food preservatives, e.g. Herbor 025[®] and Spice Cocktail from FIS S.A.⁹ and Herbalox[®] Seasoning from Kalsec[®].

⁹ FIS S.A. was a subsidiary of Nestlé, but they sold the rights to Herbor 025[®] to a French company, Robertet, and then were sold themselves to Givaudan in January.

Aruoma's team demonstrated that both products, which contained carnosol and carnosic acid, had strong antioxidant properties in the Rancimat test, and also inhibited HIV infection at low concentrations [Aruoma *et al.* 1996]. As Bauman's group elucidated, there are numerous advantages to using natural antioxidants: higher efficiency, GRAS for all uses, no restrictions on ingestible quantities, labels with ALL NATURAL as a marketing advantage, no limits on use [Bauman *et al.* 1999]. Though the production costs are higher than those of synthetic antioxidants, these costs are "negligible" compared with the final prices of food products [Bauman *et al.* 1999]. There is some question about this theory, at least in the United States. One food processor¹⁰ stated that his company paid between \$1 and \$2 per pound for BHA and would not consider buying Herbalox[®] because of price. The highest grade of Herbalox[®], Herbalox type O, costs \$20.75 per pound.

An emerging use of rosemary is as an antioxidant supplement. One well-known vendor of dietary supplements in the USA, Herbalife, produces an antioxidant pill RoseOx[®] which is manufactured by Hauser and contains 6% carnosic acid and 3% other rosemary compounds [Kuzmenko 1999]. RoseOx[®] is listed as a chemical name for carnosic acid in the CAS registry (CAS registry number 365-09-7). Kuzmenko *et al.*'s research in 1999 demonstrated that carnosic acid works as an antioxidant *in vitro* rather than through structural effects on the mitochondrial membrane of rats.

¹⁰ Edward Levine, VP, Continental Seasoning, Inc., Teaneck, NJ, personal communication.

In the last eleven years, there has been promising research with regard to the anti-mutagenic effects of whole rosemary extract, carnosic acid and carnosol. In 1991, Singletary and Nelshopp demonstrated that a 1.0% (by weight) rosemary extract from Kalsec, Inc., inhibited the initiation and promotion of 7,12-dimethylbenz[*a*]anthracene (DMBA) induced mammary tumorigenesis in female Sprague-Dawley rats by blocking *in vivo* adduct formation. Total binding was reduced by 52% and 42% from the addition of 0.5% and 1.0% respectively of rosemary extract to the animals' diet. Huang *et al.* [1994] reported similar results from the topical application of a methanol extract of rosemary leaves (3-5% carnosol) and 98% pure carnosol to mouse skin. A significant decrease in the tumor initiation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and DMBA resulted. The data indicated that the rosemary extract inhibited the covalent binding of B(*a*)P to mouse epidermal DNA. Later studies confirmed and elaborated on this mechanism. Singletary concluded that rosemary extract and carnosol stimulated the *in vivo* activity of liver glutathione-*S*-transferase (GST) and NAD(P)H-quinone reductase (QR) in the female rat. There were interesting differences between the results of the dietary administration of rosemary extract and carnosol, and the intraperitoneal injection of the same substances. Carnosol injected intraperitoneally was effective, but when it was provided in the diet, it did not enhance GST activity. On the other hand, supplementing the diet with 0.25% rosemary extract and injecting rosemary extract intraperitoneally were both effective in stimulating the activities of the Phase II enzymes GST and QR. Offord *et al.* [1995] prepared rosemary extract (15% carnosol and carnosic acid), carnosol, and carnosic acid, and found they were all

effective in preventing chemically induced carcinogenesis in cultures of human bronchial epithelial (BEAS-2B) cells by active metabolites of B[a.]P. The levels of GST π (the primary isomer of GST in the human lung) mRNA increased by three to four times after the addition of carnosol. GST π is thought to reduce DNA adduct formation in human lung cells. The mechanisms they identified were the inhibition of the expression of the phase I enzyme, CYP1A1, and the induction of the expression of the phase II enzyme GST.

In September of 2001, Dörrie's team found carnosol to be a "novel chemotherapeutic agent" against cancers that express high levels of Bcl-2, a protective protein [Dörrie *et al.* 2001]. In particular, carnosol was effective in inducing apoptosis in B-lineage leukemias

In earlier work on food preservation, carnosol was found to be more efficient than BHT or BHA in inhibiting the growth of six strains of food bacteria by Collins and Charles [1987]. Neither carnosic acid nor carnosol have been available commercially until very recently. Surprisingly, Alexis Biochemicals, the only seller, prices carnosol at sixty times the price of carnosic acid (\$60 per milligram vs. \$1 per milligram).

Depending on factors such as extraction method and plant origin, the yield of carnosic acid can be up to 7.4% of the dry weight of the starting material. Obtaining consistent and consistently high yields of carnosic acid, however, is difficult because genetic, environmental, and seasonal conditions affect the quantity of carnosic acid. Hidalgo's team stated: "High individual variability of a markedly random nature was previously reported in a study on biomass production." [Hidalgo *et al.* 1998]. It is

well known that the monoterpene content of rosemary depends on the country of origin [Svoboda and Deans 1992; Chalcat *et al.* 1993], the soil [Moretti *et al.* 1998], the collection time [Boutekedjiret *et al.* 1999], the variety [Domokos *et al.* 1995], and the method of distillation [Granger *et al.* 1973]. Chalchat's group established that, from samples of twelve rosemary oils from three different countries, there were at least two distinct chemotypes based on monoterpenoid composition: oils from North Africa contained more than 40% 1, 8-cineole, while oils from France and Spain had less than 30% of this monoterpenoid. Within Moroccan oils, there were two further subdivisions with one group containing 50-58% 1,8-cineole and the other containing 44-47% of the same monoterpenoid. In fact, the entire chemical profile of oil from each geographical source differed by percentages of the major monoterpenoids and the presence of minor constituents. Verbenone, for example, was present in both French oils, but in only one of the other nine oils. Domokos' group developed a new frost resistant variety of rosemary in Hungary in the late 1980's and identified octan-3-ol as a component (trace to 10%) of rosemary oil for the first time [Domokos *et al.* 1995].

Since diterpenoids are related compounds produced further along the isoprenoid pathway, similar factors could also cause varying carnosic acid content among different rosemary plants. In fact, Richeimer and his colleagues cited widely varying carnosic acid content for dried and wild rosemary based on country of origin, with Albanian rosemary having the lowest at 1.7%, and Moroccan rosemary the highest at 3.8% [Richeimer *et al.* 1996]. Obtaining predictable yields of the phenolic diterpenoids of rosemary is complicated by their ready oxidation as mentioned above.

Although Munné-Bosch and Alegre [2000] were sufficiently concerned about the genetic variability of carnosic acid to obtain cuttings from the same individual to root and use as progeny, they did not cite the work of Hidalgo's group [Hidalgo *et al.* 1998] who embarked on their study of rosemary because "... the variability of phenol diterpenoid contents in *R. officinalis* introduced by environmental and genetic factors remains unknown." Based on Hidalgo *et al.*'s findings, they concluded that, "Differences of up to 50% among individuals of the same population are thus to be expected." Greater variability was found among populations of wild rosemary than among populations of cultivated rosemary. They also determined that there were no morphological indicators to signal plants with different levels of carnosic acid: "... the variability seems to be of genetic origin alone."

The hypothesis that there is high variability in the concentration of carnosic acid is supported by the results of researchers cited in **Table 1.1**. The carnosic acid content of dried rosemary leaves ranges from a low of 1.10% [Munné-Bosch *et al.* 2001] to a high of 7.3% [Hidalgo *et al.* 1998]. The actual drying process can also introduce destruction of antioxidant compounds. Ibanez *et al.* [1999] found a correlation between drying temperature and antioxidant yields due to the decomposition of the antioxidant compounds. Rosemary leaves dried at 45°C in the oven had the lowest activity. Carnosol has been found at lower levels and in a narrower range from (0.02% to 1.2%). More recently, Caruso *et al.* [2000] examined the carnosic acid content of regenerated shoots of rosemary, but cited no levels, stating simply, "... quantitatively similar amounts of carnosic acid were found ..."

The work of Munné-Bosch and Alegre [2001] was the most thorough with

examination of seeds, leaves, sepals, petals, stems and roots. Caruso's team found carnosic acid in the roots of micropropagated plants, but there are no other such reports. Munné-Bosch found no carnosic acid in the roots of rosemary grown *in terra*.¹¹ A related diterpenoid, royleanone, was found in the roots by Brieskorn *et al.* in 1964.

Carnosic Acid (%)	Extraction Method	Leaf Source	Reference
1.5-2.5	Various	Unspecified	Aeschbach and Philipposian 1993
3.1-5.3	SC-CO ₂	Wild	Hidalgo <i>et al.</i> 1998
6.9-7.4	SC-CO ₂	Cultivated	Hidalgo <i>et al.</i> 1998
1.10	Liquid solvent	Cultivated	Munné-Bosch and Alegre 2001
1.7-3.5	Liquid solvent	Unspecified	Richheimer <i>et al.</i> 1996

Table 1.1 Representative concentrations of carnosic acid (expressed as percentages) from dried leaves of *Rosmarinus officinalis*, as reported in the literature.

The two groups in Spain, Hidalgo's at the University of Cordoba and Munné Bosch's at the University of Barcelona, both examined the variability in carnosic acid content due to climatic factors. Both groups hypothesized that carnosic acid performs a protective role in the plant. This was suggested in the 1992 work of Gonzalez *et al.* [1992]: "... abietatriene diterpenes in the genus *Salvia* serve as *de facto* quenching agents protecting the plants against the damage caused by singlet oxygen." Munné-Bosch and his colleagues [1999a] concluded that during periods of high stress, e.g. maximum sunshine, maximum temperature, minimal rainfall: "The large amounts of carnosic acid, alpha-tocopherol, and carotenoids present in rosemary leaves might contribute to the prevention of oxidative damage in plants exposed to drought." Both groups hypothesized that the concomitant increase in the highly oxidized diterpenoids

¹¹ Both cultivated rosemary and wild rosemary grown in soil.

reflects the fact that the carnosic acid and carnosol, less highly oxidized diterpenoids, perform a protective role as they are oxidized to derivative compounds. In fact, Munné-Bosch and Alegre's latest research [2001] pinpointed the location of carnosic acid to the chloroplasts of *Rosmarinus officinalis*. It has been known for some time that diterpenes are synthesized in plastids via the 1-deoxy-d-xylulose-5-phosphate pathway and not the classic acetate/mevalonate pathway [McGarvey and Croteau 1995]. Carnosol, rosmanol, and isorosmanol were also isolated from the chloroplast fractions, whereas 12-methoxy carnosic acid was found primarily in the plasma membrane [Munné-Bosch and Alegre 2001]. These discoveries supported the Munné-Bosch group's hypothesis that carnosic acid provides a protective function in the chloroplasts that experience the maximum oxidative stress (e.g. high light), while the *O*-methylated forms of carnosic acid and isorosmanol perform a membrane stabilization function.

Munné-Bosch and Alegre found that levels of carnosic acid, carnosol, and 12-methoxy carnosic acid varied according to seasonal stresses, specifically as a function of temperature, illumination, and rainfall [Munné-Bosch and Alegre 2000]. The levels of these three diterpenoids declined during the summer, while isorosmanol, a more highly oxidized diterpene increased and rosmanol levels were stable. As the environmental stresses, e.g. prolonged drought, moderated during the fall, the levels of carnosic acid, 12-methoxy carnosic acid and carnosol rose, reaching peaks in October (12-methoxy carnosic acid) and November (carnosic acid and carnosol). This variability, shown in **Table 1.1**, is attributable in part to seasonal conditions.

Given the increasing awareness of rosemary's role in human health, researchers have been examining ways to increase the yield of carnosic acid and to produce consistent yields. Tissue culture is an often-used technique to detect and to study the formation of natural products in plant material [Murashige and Skoog 1962]. It can also be a means to identify and propagate high yielding clonal lines [Yang *et al.* 1997]. The use of tissue culture as a means to study and enhance the production of these important compounds is potentially valuable. The advantages of growing cultures in the laboratory rather than in the ground include independence from variations due to seasonal conditions, manipulation of culture conditions to alter yields, production of novel compounds due to unique culture conditions (e.g. 100% humidity). In addition, culture conditions can be created and modified: solid media for callus or micropropagation, cell suspension cultures, shoot regeneration from explants, shoot regeneration from callus, cell free extracts from callus culture. In particular, the use of liquid media for the growth or maintenance of tissue provides an unusual environment where the use of elicitors and/or traps can be effective in the enhancement of yield [Basile *et al.* 1993].

There is evidence that monoterpenoids were produced by shoots regenerated from rosemary callus [Webb *et al.* 1984], and that monoterpenoids were produced by rosemary callus [Tawfik 1992]. Caruso's results indicated that carnosic acid was present in regenerated shoots of rosemary. It was surprising, however, that in their paper published in 2000, they reported "Quantitatively similar amounts of carnosic acid were found in samples from seed grown plants, green callus and regenerated specimens."

The intent of the research for this dissertation was to use tissue culture as a way of investigating the existence and levels of carnosic acid and related diterpenoids in the undifferentiated cells and regenerated plants of rosemary. The study consisted of two parts: (1) tissue culture of rosemary to produce callus and to regenerate plants and (2) extraction, isolation and analysis of phenolic diterpenoids from those tissues and tissues of cultivated rosemary.

The major findings of this research, as reported in the following two chapters, are:

- Callus and micropropagated shoots of rosemary grown on MS media supplemented with BAP contain carnosic acid and related diterpenoids, though at significantly lower yields than the parent tissue.
- The chemical profiles and the yields of the main antioxidant compounds in callus vary with the hormonal additions to the media.
- The compounds isolated from rosemary demonstrate high antioxidant activity in the DPPH assay.

During the course of this research, several protocols were either developed or significantly modified:

- a straightforward, relatively rapid protocol for the micropropagation of rosemary was devised.
- high temperature and high light levels were found to be effective in inducing micropropagation and enhancing growth of callus and regenerated plants.
- a protocol for the extraction of rosemary shoots was adapted to the extraction of callus and of media.
- a protocol for the crystallization of carnosic acid was elucidated.

CHAPTER TWO

The Tissue Culture of *Rosmarinus officinalis*

SUMMARY

The addition of high levels of BAP to MS basal media with IAA and kinetin and the maintenance of cultures at elevated temperature and high illumination have resulted in the relatively rapid regeneration of rosemary plants with one long initial passage and no required changes in media formulation. This labor saving protocol could be useful in regenerating rosemary for phytochemical and morphological investigations. Two different media formulations were found to produce healthy green or white callus. Elevated temperatures and light levels enhanced the growth of regenerated shoots, while lower light and temperature levels ensured the maintenance of callus.

Introduction

In the realm of tissue culture, a field now almost 100 years old, the interest in rosemary has been relatively recent and the literature relatively sparse. Rosemary is difficult to grow from seed, but extremely easy to grow through divisions or cuttings, so there has been little benefit of tissue culturing rosemary for horticultural purposes. On the other hand, the natural products from rosemary, specifically the essential oils, rosmarinic acid and the phenolic diterpenoids, are being increasingly recognized for their human health benefits. Tissue culture is an ideal medium through which to study the formation of these products. Additionally, rosemary plants found in the wild or raised in natural settings are subject to wide variations in natural product compositions and yields, largely as a result of varying and unpredictable climatic, edaphic and geographic conditions as described in Chapter One. Inherent in the tissue culture process is control over growth and maintenance conditions. Light, temperature, nutrients, and hormones can all be manipulated by the researcher to affect a desired outcome.

The first reports of rosemary in tissue culture were published in the same year, 1984. Chaturvedi *et al.* [1984] are correctly credited with the first paper on the micropropagation of rosemary, but Webb's group was the first to report on callus of rosemary in tissue culture and the regeneration of shoots from callus [Webb *et al.* 1984]. Chaturvedi and Misra's interest arose from concerns about the well-known variability in the content of rosemary essential oil and a desire to clone rosemary for

uniform oil production. Webb's group had a more theoretical interest in determining if organogenesis were necessary for the formation of essential oils. A bit later, Cristea and Cachita-Cosma were similarly less interested in the practical applications of tissue culture than in the morphological behavior of a variety of rosemary explants on different media and hormonal formulations [Cristea and Cachita-Cosma 1991].

More recently, Yang *et al.* [1997] studied rosemary in tissue culture, with a focus on enhancing the production of rosmarinic acid, an antioxidant, through clonal multiplication. Lastly, Caruso *et al.* recently reported the detection of carnosic acid in regenerated shoots of rosemary [Caruso *et al.* 2000]. Unlike the previous report of carnosic acid in "*in vitro*-grown" shoots of *Salvia canariensis* by Luis *et al.* [1992], the shoots in Caruso's research were produced after a dedifferentiation phase. The shoots of *S. canariensis* appear to have been adventitious shoots, hence the existence of carnosic acid is not proof of *de novo* synthesis. Caruso *et al.* also reported that carnosic acid was present in green callus of rosemary, but this was nodular callus so this was not evidence that undifferentiated cells contained carnosic acid.

Between 1984 and 1991, Misra and Chaturvedi's group published five papers describing a basic protocol and nutrient and hormonal variations to enhance the *in vitro* multiplication of adventitious shoots of rosemary from aseptically grown shoot apices [Chaturvedi *et al.* 1984; Misra and Chaturvedi 1984, 1987; Jain *et al.* 1991; Misra and Chaturvedi 1991]. Similarly, Luis *et al.* in 1992 reported working with "plantlets", but given that these plantlets appeared in seven days, they were adventitious shoots arising from the apical bud explant starting material. Yang *et al.*

[1997] started with heterozygous seeds for the propagation of clonal lines of high rosmarinic acid producing plants.

Typically, working with rosemary in tissue culture has involved multiple, complex steps over a long period. Rodney Croteau, one of the foremost investigators of terpenoids in tissue cultured Lamiaceae genera (e.g. *Salvia*, *Mentha*), once commented (personal communication May 1995) that though rosemary was of interest to his group, they found it too difficult to regenerate. The sources of difficulty in producing rosemary callus only include identifying productive explant material, reducing high rates of infection from *in terra* explant tissue, determining optimal levels and combinations of hormones and adequate levels of macro and micronutrients. Chaturvedi, Misra, and Sharma [1984] reported the almost total decimation of shoot apices and nodal segments as explants so they used axillary buds to establish aseptic plant material. They in turn cut shoot apices from the aseptic plants to use as starting material. Yang *et al.* [1997] modeled their approach on that of Chaturvedi *et al* [1984], but started with heterozygous seeds, which were relatively easy to sterilize thoroughly. Cristea and Cachita-Cosma found that shoot apices produced callus only and stem segments showed no response at all. They also found, as Tawfik did in 1992, that nodes produced a variable response, either callus or shoots, depending on the media [Tawfik 1992].

Regenerating entire plants from rosemary explants has been significantly more difficult and complicated than obtaining either callus or regenerating shoots. Webb *et al.* (1984) and Tawfik (1992) found that shoots could be regenerated from callus with a change in the media. Webb *et al.* (1984) reported successfully regenerating shoots from callus within four weeks of transferring the callus from Murashige and Skoog (MS) media to the same basal media supplemented with 20mg/L of benzylaminopurine (BAP) and changing the

photoperiod. Tawfik was also successful in producing shoots on MS media with added benzyladenine (BA). Inducing root formation in regenerated shoots, however, proved impossible for Webb, and required a transfer out of the tissue culture system for Tawfik. Caruso *et al.* [2000] reported the regeneration of plants with three changes in media and more than a twenty-one week gestation period.¹

Although the effects of temperature and radiation levels on rosemary in the field have been studied recently by Munné-Bosch *et al.* [1999a, 1999b, 2000] and Nogues and Baker [2000], there has been only one report [Banthorpe *et al.* 1986] of light and temperature effects on rosemary in tissue culture, and this examination related only to growth and pigmentation of callus.

Since the regeneration of rosemary plants reported in the literature has involved frequent passages and/or changes in media to produce rooted shoots, there seemed to be a need for a protocol with fewer and simpler steps. This paper describes a straightforward, relatively rapid protocol for producing plantlets from greenhouse grown *Rosmarinus officinalis* that employs a single nutrient-hormone formulation using standardized media, infrequent passages and uniform conditions for incubation, with concomitant savings in time and labor. The developmental effects of different media treatments, which were observed during the course of this investigation, are also reported. During the course of devising an efficient micropropagation system, positive effects of elevated temperature and light conditions on the growth of callus and plantlets were identified and will be discussed.

¹ Caruso *et al.* reported an initial 6 week culture period, followed by a second 6 week period during which shoots formed, then an unspecified period during which roots formed, and, lastly, a nine week period during which the shoots grew to 8 cm.

MATERIALS AND METHODS

Plant Material. Cuttings from rosemary plants from the Lehman College greenhouse and research beds were used as explants for the experiments. Four cultivars of rosemary were purchased in 1994 and 1998 from the Peconic River Herb Farm, Calverton, New York: *Rosmarinus officinalis*, *R. officinalis* var. Arp (a frost resistant variety), *R. officinalis* var. Salem and *R. officinalis* var. prostratus. The *R. officinalis* var. Arp was maintained in the research beds year round, and two of the *R. officinalis* plants remained in the greenhouse year round along with the micropropagated plants. The other rosemary plants were moved from the greenhouse to the research beds every May and returned to the greenhouse every October.

Innoculum preparation. Shoot cuttings, measuring 8 cm, were removed from all three varieties plus the species, but due to high rates of infection² of the three varieties, only the species was used for tissue culture. The cuttings were surface disinfected in 95% ethanol for thirty, sixty, or ninety seconds. Further sterilization was achieved by soaking and constantly shaking the cuttings for ten minutes in 300 ml of 15% (v/v) sodium hypochlorite (appropriately diluted Clorox™), containing 0.1% w/v Alconox detergent as a surfactant. The shoots were then washed by rinsing three times with sterile, distilled water in a laminar flow hood. Shoot tips, leaf blade sections and nodal segments with leaf bases attached, each measuring 5 mm, were excised from the shoot cuttings in sterile petri dishes. The inocula

² Rates of infection (within one week of inoculation): *R. officinalis* 0%, *R. officinalis* var. Salem 27%, *R. officinalis* var. prostratus 40%, *R. officinalis* var. Arp 63%, (n=15). In five subsequent experiments (n=20), *R. officinalis* became infected at an average rate of 13%.

were placed basal end down, two or four per jar, in 100 ml glass baby food jars containing 30 ml media, covered with clear Magenta[®] caps and sealed with Parafilm.

Solid Media Formulations.

Gamborg's B-5 basal medium Gamborg's B-5 basal medium [Gamborg *et al.* 1968] with minimal organics (Sigma) was supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L 6-benzylaminopurine (BAP) and 2.0% w/v sucrose. Murashige Skoog basal medium (Sigma) was supplemented with 1 mg/L kinetin, 10 mg/L indoleacetic acid (IAA), either 0, 2, 4, or 6 mg/L BAP and 3.0% w/v sucrose. The culture media was adjusted to pH 5.80 +/- .01 with 0.1 N or 1 N NaOH or HCl.

Chaturvedi and Misra's Media. The media formulations devised by [Chaturvedi *et al.* 1984] for the induction of shoot bud differentiation in nodal stem segments were used with two modifications: nodal segments of greenhouse material were used as explants and only the rooting media formulation with IAA was used.

Murashige Skoog Media. The media formulations recommended in Murashige's 1962 paper were followed with 10 mg/L of IAA and 1 mg/L of kinetin selected as concentrations of these two hormones from the ranges proposed in the paper. For plant regeneration experiments, 0,2,4 or 6mg/L BAP were added. For the factorial experiment, the levels of BAP and 2,4-D are specified in **Table 2.2**.

The three media formulations are compared in **Table 2.1**: the original Murashige and Skoog [Murashige and Skoog 1962] medium (citing ranges for IAA and kinetin), the modified MS actually used with 10 mg/L IAA, 1 mg/L kinetin and supplemental BAP, and the Chaturvedi and Misra [Chaturvedi *et al.* 1984] media. Note that agar specified by Murashige and Skoog was replaced by gellan.

Media		MS original	MS modified	CHAT
Units		mg/L	mg/L	mg/L
Macro Nutrients	(NH₄)NO₃	1650.0	1650.0	1000.0
	KNO₃	1900.0	1900.0	1500.0
	CaCl₂ 2H₂O	440.0	440.0	0 or 50
	MgSO₄ 6H₂O	370.0	370.0	200.0
	KH₂PO₄	170.0	170.0	80.0
Micro Nutrients		same	same	same
Iron compounds	Na₂ EDTA	33.6	33.6	22.4
	Fe₂ SO₄ 7H₂O	27.8	27.8	16.7
Vitamins	Glycine	2.0	2.0	2.0
	Nicotinic Acid	0.5	0.5	0.5
	Pyridoxin HCl	0.5	0.5	0.5
	Thiamin HCl	0.1	0.1	0.1
Hormones	IAA	1 to 30	10.0	0.1
	Kinetin	0.04 to 10	1.0	0.0
	BAP	0.0	0,2,4,6	0.3
	AdS	0.0	0.0	10.0
	Inositol	100 mg/l	100 mg/l	0.0
	Malt Extract	0.0	0.0	50.0
Units		g/l	g/l	g/l
Agar		10.0		7.0
Gellan			4.5	
Sucrose		30.0	30.0	30.0
pH		5.7 to 5.8	5.7 to 5.8	5.8

Table 2.1 Comparison of hormone and nutrient formulations as proposed by Murashige and Skoog [1962], Chaturvedi *et al.* [1984], and the final formulation used for plant regeneration.

Multifactorial experiment. Sixteen different combinations of 2,4 dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) were used (**Table 2.2**). Five cultures were inoculated for each of 16 hormone combinations.

2,4-D \ BAP	0.225 mg/L	1.125 mg/L	2.250 mg/L	11.25 mg/L
0.055 mg/L	A1	A2	A3	A4
0.110 mg/L	B1	B2	B3	B4
0.550 mg/L	C1	C2	C3	C4
1.100 mg/L	D1	D2	D3	D4

Table 2.2 Concentrations of BAP and 2,4-D used for the factorial experiment.

Before heat sterilization, 4.5% w/v Gellan (Research Organics, Inc., Cleveland, Ohio) was added and heated until dissolved. The media were distributed at a rate of 30 ml/jar into 100 ml “baby food jars”, closed with Magenta caps and autoclaved.

The inocula were incubated under 1200 lux at a 16-hour photoperiod, at either 25.0°C +/- 1.0°C or 28.0°C +/- 1.0°C. The calli were subcultured after two, four, six or nine weeks by cutting each cluster into four pieces and transferring the pieces, two per jar, to new jars with the same media. Where shoots and/or roots developed, the shoots with and without roots were separated from the calli. The shoots and/or plantlets were transferred to new jars with the same media. After six more weeks, the plantlets were either subcultured again or removed from the culture jars and transferred to soil. For maintenance in tissue culture, two media formulations were used: MS basal media with 10 mg/L IAA and 1 mg/L kinetin,

supplemented with either 0 or 4 mg/L BAP. For transfer to soil, the plantlets were first rinsed to remove the gellan and clipped to a uniform height of 5 cm. They were then transferred to 4" plastic pots containing sterilized, water-saturated Metromix; the pots were covered with plastic bags and placed in a glass aquarium tank with dampened capillary matting. The chamber containing the tanks was maintained at 24.0°C +/- 1°C under a 16-hour light/8 hour dark photoperiod, with light at 1200 lux, and relative humidity at 80%. After two weeks, the plastic bags were taken off and four weeks later, the plants were moved into the greenhouse.

Light and Temperature Experiment. The inocula were incubated in two double door (Perceival) culture chambers both set at a 16-hour light/8 hour dark photoperiod, one set at 25°C and the other at 30°C. Each chamber was divided into two areas with different light intensities (**Table 2.3**). One side of each chamber was kept at a low intensity of 300 lux, and the other was kept at 1200 lux under Philips white light bulbs. A black cardboard was used to separate the two light intensities. To achieve light intensity of 300 lux, a piece of aluminum foil was used to increase the light intensity and keep it constant at 300 lux. Four bulbs were used for high light intensity and two light bulbs were used for low intensity.

300 lux at 10°C	1200 lux at 10°C
300 lux at 25°C	1200 lux at 25°C
300 lux at 30°C	1200 lux at 30°C

Table 2.3 Experimental conditions to test effects of three sets of temperature and illumination levels on growth of tissue cultured callus and plantlets of rosemary.

Liquid Media Formulations. Shake cultures were initiated from stock callus cultures on solid media (see descriptions of Gamborg's B-5 and Murashige Skoog above under **Solid Media Formulations**). Stock calli were subcultured every four weeks. Shake cultures were maintained in the dark in one liter Erlenmeyer flasks with clear plastic closures, containing 200 ml heat-sterilized aqueous medium of the same composition as the stock cultures and inoculated with an average of 5.5 grams fresh weight callus tissue. The flasks were shaken on gyrotory shakers (New Brunswick G-10) set for 125 rpm at a temperature of $25.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$. Evaluation by TLC of the B-5 media indicated the absence of phenolics so use of this media was discontinued.

RESULTS

Optimal nutrient and hormone combinations for plant regeneration and callus initiation and maintenance for rosemary in tissue culture were developed. During the course of these studies, two very different combinations resulted in the formation of healthy callus with, however, different phenotypes: green callus and white callus. A protocol was also developed for the relatively rapid regeneration of rosemary in tissue culture using one media formulation throughout the culture cycle. Lastly, preliminary conclusions were drawn about the positive effects of high light and high temperature on the growth of rosemary callus and shoots.

Plant Regeneration

In the first set of experiments to regenerate plants, the media formulations described by Chaturvedi *et al.* [1984] produced similar results to those reported. However, when the growth results of the Chaturvedi protocols were set side by side with the results of using MS with 2,4, or 6 mg/L BAP, the Chaturvedi protocols were less productive. In one set of experiments (n=16), 50% of the inocula died after the second subculture. The remaining cultures all had shoots, but these were smaller on average than those in the MS/BAP media. As suggested by Chaturvedi *et al.*, calcium was removed from the media in another experiment to induce greater shoot development. In this case (n=40), 58% of the inocula developed shoots, but this was still a lower rate than on MS/BAP. A key time related problem with the Chaturvedi protocols was the requirement to make up a non-standard medium, whereas MS was available in packets thus saving considerable time and effort. Lastly, the Chaturvedi protocol was predicated on micropropagation via adventitious shoots.

In this approach, there is no intervening callus stage so that for phytochemical purposes, it is difficult to determine if the detected natural products are synthesized *de novo* or simply carried over from the parent tissue. Luis' group, which has reported extensively on diterpenoid production in *Salvia* species, reported the presence of carnosic acid in cultured shoots of *S. canariensis* [Luis *et al.* 1992]. They induced axillary buds on Almacigo's medium and multiplication of those buds on modified MS medium, but there was no intervening callus stage. The advantage of using the full strength MS and additional BAP is that the explants dedifferentiate into a mass of cells first and many more than two shoots per node regenerate from the callus.

To induce effective plant regeneration, two factors were identified: the presence of BAP and the length of the subculturing intervals. The addition of 2, 4, or 6 mg/L of BAP to Murashige Skoog basal media (with 10mg/L IAA and 1 mg/L kinetin) caused shoot formation within four weeks. Although the lowest concentration of BAP, 2 mg/L, produced plantlets in 50% of the explants (duplicate experiments, n=15), the number of shoots was one third of the number produced by the media with 4 mg/L of BAP. The 4 and 6 mg/L concentrations of BAP produced almost equal numbers of shoots, so the lower amount was selected for routine use.

The photographs in **Figures 2.1 - 2.6** depict the six key stages of development from explant to regenerated plant on media supplemented with 4 mg/L of BAP. There was a steady development from some shoots (29%) at seven weeks to primarily shoots (72%) at 10 weeks to primarily rooted shoots (62%) at 17 weeks. The development from ten weeks to seventeen weeks is shown in **Figure 2.7**.

Figure 2.1 First appearance of callus on nodal explant of rosemary two weeks after inoculation.

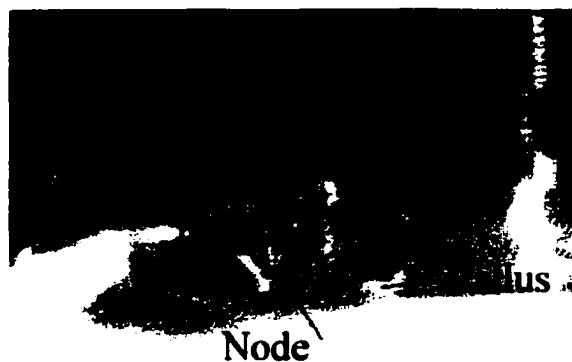


Figure 2.2 Callus enveloping explant four weeks after inoculation.

Figure 2.3 Shoot emerging from callus six weeks after inoculation.



At seventeen weeks the plants were transferred to soil, moved to maintenance media (MS without BAP), or subcultured once more on media supplemented with BAP.

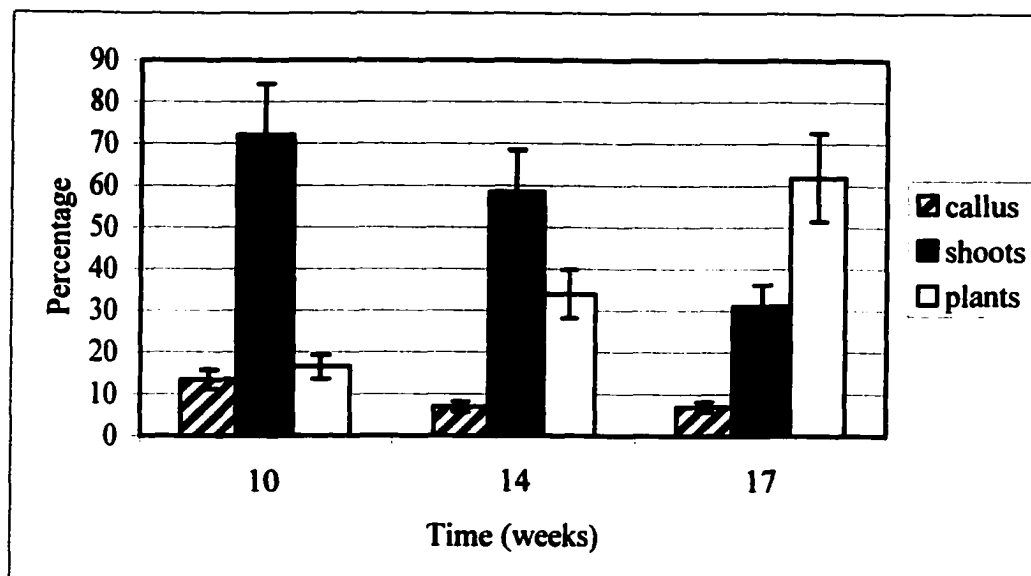


Figure 2.7 Development of regenerated plants at 28°C from 10 weeks to 17 weeks. Percentages expressed as means of triplicate experiments +/- standard error, n=5.

After 6 weeks on MS media with 4 mg/L BAP, the average number of shoots per inoculant was 3.2, while after 13 weeks the average increased to 4.2 (duplicate experiments, n=5). The maximum number of shoots at seventeen weeks was 12 from one original explant. When healthy shoots were moved to either hormone free media or media with only auxin or only cytokinin, growth was poor. In MS media, with no added hormones, roots were produced in 30% of the inocula, but the tissue was unhealthy and browned quickly.

Subculturing initially at intervals of fewer than four weeks resulted in shoot proliferation, but minimal root development. When the initial passage was lengthened to nine weeks, roots emerged at the end of the first culture period or within a week of subculturing. Thereafter, subsequent passages were reduced to less

than six weeks for plant development. When the second passage was longer than six weeks, 90% of the plantlets became brown and died.

Leaving the inocula undisturbed for an initial nine-week period was a condition to induce root formation without a change in the media. When subculturing was performed more frequently (at four or six week intervals), using MS basal media with added BAP, roots did not form. Endogenous auxin affects root formation in tissue culture [Dodds and Roberts 1993], either as an inhibitor [Banthorpe *et al.* 1986] or as an inducer, depending on the interaction between endogenous and exogenous auxin. In these rosemary cultures, exogenous auxin, in the form of IAA, was presumably exhausted during the nine-week initial culture period. Root initials may be formed as the exogenous supply declines at the end of this period since they appear either just before or immediately after subculturing on fresh media.

Preliminary Temperature Studies

Temperature and light levels are factors in the growth of callus and also in the rapid regeneration of plants from callus tissue. A comparison of shoot and root development under two different temperatures (25°C and 28°C) showed that plantlets developed at both 25°C and 28°C, but that the rate of formation at the higher temperature was more than four times that at the lower temperature (**Figure 2.8**). At the higher temperature, 62% of the inocula developed into plants, while at the lower temperature only 16% did.

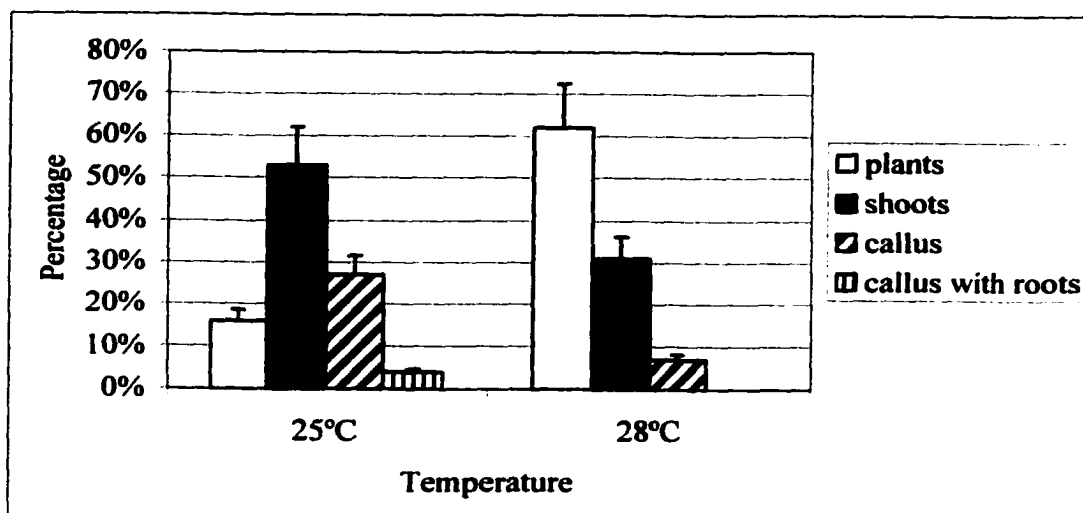


Figure 2.8 Developmental differentiation (percentages, expressed as means plus standard error) at two different temperatures 17 weeks after inoculation. Triplicate experiments, $n=10$.

At the lower temperature, there was also a detectable level of reversion to callus, which did not occur at the higher temperature.

Light and Temperature Experiments

The observations from the preliminary light and temperature studies led to the design of a more complex experiment based on three temperatures and two radically different light levels. The immediate result of this six-part experiment was that 10°C was determined to be too low a temperature for growth of rosemary tissue, so the experiments for this temperature at the two light levels were discontinued. Most of the studies of field grown rosemary cited local temperatures within several degrees of 25°C. Although *Rosmarinus officinalis* is hardy above 0°C, 10°C is obviously too extreme for growth.

At both temperatures at the lower light intensity (300 lux), callus predominated. At the higher light intensity (1200 lux), plantlets predominated. **Figures 2.9 and 2.10** show the proliferation of shoots at the higher light level (1200 lux) at both temperatures (25°C and 30°C). Plantlets grew larger at 30°C than at 25°C. **Figures 2.11 and 2.12** are photographs of

the calli at the lower light level. At 25°C, the calli are small with no visible shoots; at 30°C, the calli are larger, but still show no evidence of shoot development. Based on the height and weight results (Figures 2.13 and 2.14), the higher illumination appears to be a stronger factor than the higher temperature. Cultures grown under 25°C and 1200 lux light intensity had small shoots compared with the 30°C and 1200 lux.

At the early stages, the cultures maintained at 25°C grew more slowly than those grown at 30°C did. Measurements of the greatest height and greatest width of these cultures showed that the results at 25°C and 30°C were somewhat similar. However, the cultures maintained at 30°C had a head start in the early development stages and were thus still slightly larger than the cultures maintained at 25°C.

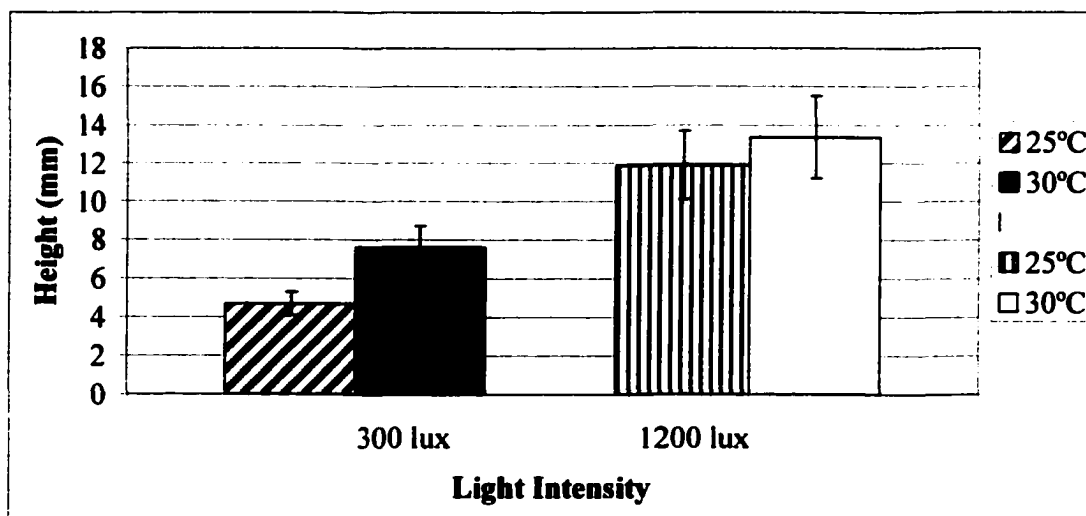


Figure 2.13 Comparison of heights (mm) of 8 week old calli and plantlets at two light intensities (300 and 1200 lux) and two temperatures (25°C and 30°C).

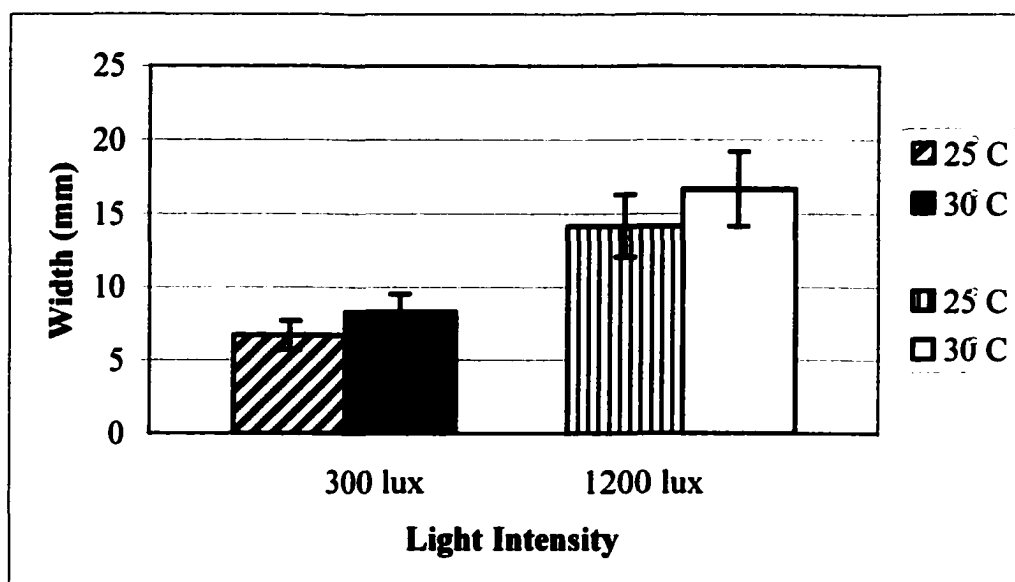


Figure 2.14 Comparison of widths (mm) of 12 week old calli and plantlets at two light intensities (300 and 1200 lux) and two temperatures (25°C and 30°C).

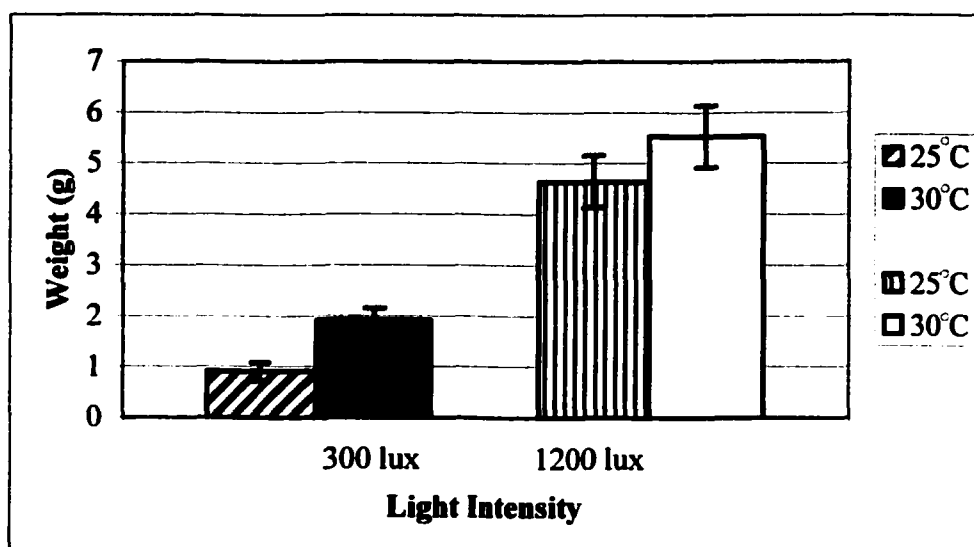


Figure 2.15 Comparisons of weights (g) of 10 week old calli and plantlets at two light intensities (300 and 1200 lux) and two temperatures (25°C and 30°C).

Callus Production

Healthy green and white calli were sustained by subculturing at four-week intervals. Leaving the calli longer produced necrosis. On Gamborg's B-5 medium with 2.0% w/v sucrose, callus began to form within three days of inoculation, was white, even under light, and remained morphologically undifferentiated through one and a half years of subculturing. Green callus was produced on MS media with 10 mg/L IAA and 1mg/L kinetin and 3.0% w/v sucrose within one week of inoculation. Nodes, shoot tips and midsections were used as explants. In thirty-four percent of the inocula, roots emerged from the green callus (n=15, triplicate experiments) within six weeks. After eleven weeks, the only notable difference was that roots were formed on the callus in 66% of the nodal explants and 86% of the shoot tip explants. After sixteen weeks, 100% of the inocula had formed callus and 92% had formed roots, but only 25% produced shoots. Transferring the callus with roots to media

supplemented with BAP increased the rate of shoot formation slightly. Both sets of calli were maintained for one and a half years.

Multifactorial experiment

The intent of the multifactorial experiment was to determine if there was a correlation between phenotype and chemical composition of calli. Two gradients were created by varying BAP and 2,4-D concentrations. There are reports in the literature of alterations in the media either affecting the terpenoids constituents of plants grown *in vitro* or of increasing yield [Nabeta *et al.* 1983; Miyasaka *et al.* 1986; Banthorpe *et al.* 1990; Segura and Calvo 1991; Knoss 1994, 1995]. The differing concentrations of 2,4-D and BAP had significant morphological effects almost immediately as shown in **Table 2.4** and shown in the photos in **Figures 2.16 – 2.19**.

BAP \ 2,4-D	0.225 mg/L	1.125 mg/L	2.250 mg/L	11.25 mg/L
0.055 mg/L	green/cream	green	green/cream	green/cream
0.110 mg/L	green/cream	green	yellow	green
0.550 mg/L	cream/brown	green/cream	cream/brown	cream
1.100 mg/L	brown	green/cream	brown	brown

Table 2.4 Phenotypical effects of different concentrations of BAP and 2,4-D on callus of rosemary.

By observation, the callus with the higher concentrations of 2,4-D (1×10^{-6} M and 5×10^{-6} M) produced smaller, less healthy calli. Based on their fragile condition, these calli were not subcultured

DISCUSSION

Obtaining green callus from rosemary explants on MS solid media was expected given the report by Webb *et al.* 1984, but the hormonal composition had to be worked out. Webb's group used modified MS media based on the original paper by Murashige and Skoog, but they did not specify the exact amounts of IAA and kinetin within the ranges suggested by Murashige and Skoog. They also supplemented the MS media with 10% coconut milk initially and 2% after the first subculture. The addition of 10 mg/L of IAA, 1 mg/L of kinetin and 3.0% w/v sucrose produced healthy green callus that, with subculturing at four-week intervals, remained morphologically undifferentiated for one and a half years. This tissue was later extracted and the extracts tested for the presence of antioxidant diterpenoids, as described in Chapter Three.

Webb *et al.* were also successful in regenerating shoots from two year old callus by adding an unspecified amount of BAP to the media and moving the callus from natural daylight to a 16 hour light / 8 hour dark photoperiod, however, they were unable to regenerate plantlets. Cristea and Cachita-Cosma [1991] were successful in inducing roots in shoot tissue, but only with alterations to the media formulation and culture conditions; they substituted indolbutyric acid (IBA) for naphthalene acetic acid (NAA) and added gibberllic acid (GA3). The shoots also had to be exposed to a dark period. Tawfik also found that a complex series of steps was required to regenerate entire plants. The rosemary shoot tip explants were initiated on MS media with 2 mg/L thidiazuron (TDZ) and 0.5 mg/L IAA to generate callus, then the callus was excised from the shoot tips and subcultured onto regeneration medium with supplemental BAP and lastly, the resulting shoots were transferred to pots filled with perlite and placed in the greenhouse under a misting system. In replicating

these experiments, the use of TDZ did not enhance callus enlargement as Tawfik had found and misting regenerated shoots in the greenhouse had too low a success rate (10%, n=50) to be viable.

The light and temperature experiment showed that growing rosemary in tissue culture under a light intensity of 1200 lux and a temperature of 30°C produces plantlets. Shoots grew quickly and well under the 30°C temperature and 1200 lux light intensity. The average heights and widths of the cultures under this light intensity and temperature were greater than those at the other light intensities and temperatures under observation. Most researchers report using a light intensity between 300 and 600 lux, and the typical temperature is 25°C. Webb *et al.* [1984], who were unable to generate roots on shoot tissue, used a similar media formulation (MS with BAP) and high light (1500 lux), but they maintained the cultures at 25°C and also subcultured at frequent (four-week) intervals.

For callus production, the lower light intensity of 300 lux is suggested by these experiments. At this low light intensity, rosemary cultures had no shoots or roots. To maximize the size of the callus in the shortest time under this light intensity, cultures should be grown at 30°C instead of 25°C.

CHAPTER THREE

Phenolic Diterpenoids from Rosemary Cultured *in vitro* and *in terra*

SUMMARY

Four phenolic diterpenoids, carnosic acid, carnosol, rosmanol, and 12-methoxy carnosic acid, were extracted from shoots and flowers of soil grown rosemary as well as from rosemary callus and regenerated shoots grown in tissue culture. Extracts containing detectable levels of these compounds were obtained by devising improved isolation procedures, including a protocol that resulted in carnosic acid being the major component of the extracts. The ability to obtain relatively high proportions of carnosic acid facilitated the crystallization of this powerful antioxidant compound.

INTRODUCTION

The successful extraction of phenolic antioxidant compounds from rosemary by chemists, food scientists, and cancer researchers over the last fifty years has required creativity and diligence. For centuries, rosemary was simply extracted either in heated water or in alcohol for medical use or added to food during the cooking process to prevent lipid peroxidation. Steam distillation became the primary commercial method for extraction of aromatic rosemary oil, which is valuable in the soap, cosmetic, and perfume industries. For food preservation, progress was made in the nineteen fifties, when complex organic solvent extraction was first applied to the extraction of rosemary. In 1955, two researchers in Yugoslavia, Rac and Ostric, reported developing an antioxidant mixture using rosemary, citing the costs of storing large quantities of foodstuffs during World War II [Rac and Ostric 1955]. They noted American industrial advances, dating from the 1920's, as models for their work and the recent discovery of rosemary's food preservation properties by Chipault's group in 1952. Rac and Ostric's requirements for effective antioxidants still apply today: the product had to be colorless, odorless, and tasteless, lipid soluble, non-toxic, cost effective to produce, and active at low doses. Their rosemary extract qualified in all these regards, especially low cost, since the plant was endemic to Yugoslavia. Research scientists in industry, government and academia have expanded on these beginnings by developing sophisticated solvent [Tena *et al.* 1997; Basile *et al.* 1998] and mechanical [Aeschbach and Rossi 1995] extraction procedures and separation techniques [Ibanez *et al.* 2000; Saénz-López *et al.* In press] and developing

commercial rosemary extracts [Kuzmenko 1999; Kuzmenko *et al.* 1999]. Cancer and nutrition researchers have refined basic extraction and isolation techniques [Ho *et al.* 1994; Huang *et al.* 1994] and used commercial antioxidant mixtures [Schwarz and Ternes 1992a; Cuvelier *et al.* 1996] to test the now well known¹ antioxidant and anti-mutagenic properties of rosemary.

Since carnosic acid is the phenolic diterpenoid in rosemary with the highest antioxidant activity, the goal of most extraction protocols is to produce an extract that consists primarily of carnosic acid. Some research suggests that the synergy of a mixture of compounds is sometimes more powerful than the effects of one single compound. In particular, Singletary's group theorized that carnosol might be more bioavailable as part of an extract than in isolation [Singletary and Nelshoppen 1991]. They also found that the extract was effective in stimulating the activities of two phase II enzymes, which protect against chemical carcinogenesis.

Whether researchers have sought to isolate carnosic acid or to produce an extract of which it is the primary component, there have been problems to overcome. Carnosol was first isolated from plant material, *Salvia carnosia* (Dougl.) more than sixty years ago [White and Jenkins 1942], but carnosic acid was not discovered until 1964 in *Salvia officinalis* [Linde 1964] and a year later in *Rosmarinus officinalis* [Wenkert *et al.* 1965]. The latter discovery occurred in the course of determining the structure of rosmarinine, "an alleged alkaloid" of rosemary [Wenkert *et al.* 1965]. Though numerous groups extracted antioxidant compounds from rosemary in the nineteen-eighties, it was not until 1993 that carnosic acid was isolated and purified by

¹ The most recent issue (volume 27, number 4, February 2002) of the Harvard Medical School, *Harvard Health Letter*, cited recent research showing rosemary as one of 8 foods and spices with high "oxygen radical absorbance capacity" (ORAC).

a group in Slovenia [Paris *et al.* 1993]. Nestec Ltd., the Swiss research arm of Nestlé, had several patents pending between 1988 and 1993, but the actual patents were not issued until October 1993 and September 1994 [Aeschbach and Philipposian. 1993, 1994].

The difficulties in isolating carnosic acid stem partly from the now established fact that “native” levels of carnosic acid in rosemary are highly variable (Table 1.1, Chapter One), with differences of up to 50% between individuals of the same population [Hidalgo *et al.* 1998]. In addition, there are conflicting reports in the literature about the optimal time to harvest rosemary for maximum yield of carnosic acid. Munné-Bosch’s group determined that carnosic acid declined by 50% during the Mediterranean summer, while Hidalgo’s group identified July as the time of peak carnosic acid production [Hidalgo *et al.* 1998; Munné-Bosch *et al.* 1999b; Munné-Bosch and Alegre 2000]. Extensive research by the Munné-Bosch group showed a correlation between drought, high light, and a decline in carnosic acid content.

In the laboratory, there are other problems to consider. Carnosic acid degrades easily to derivative compounds in polar solvents [Wenkert *et al.* 1965; Schwarz and Ternes 1992b; Okamura *et al.* 1994], in oxygen [Schwarz and Ternes 1992b] and in the presence of iron and copper ions [Richheimer *et al.* 1996]. As early as 1965, Wenkert *et al.* found that carnosic acid in methanol (150mg/5 ml), left in the dark at room temperature for 3 weeks, yielded crystals of carnosol. Schwarz and Ternes [1992b] determined that carnosic acid in methanol remained stable in daylight, when oxygen was absent. When oxygen was present, even in the dark, carnosol, rosmanol, epirosmanol, and 7-methyl epirosmanol were formed within five hours

[Schwarz and Ternes 1992b]. Although there is conflicting evidence about carnosic acid's stability at room temperature in the light, the over-riding factor seems to be oxygen. Okamura *et al.* [1994] found that carnosic acid decreased at room temperature and in the light in either acetone or as a component of an acetone extract. They concluded that "raising temperature and light tend to promote the degradation of carnosic acid" [Okamura *et al.* 1994], but they did not specify any duration or mention the presence or absence of oxygen. Their data indicated that carnosic acid declined and carnosol increased at a higher rate at 24°C than at 4°C in the dark. In the light, carnosic acid declined more than in the dark and carnosol remained stable. However, Schwarz and Ternes' results cited above indicated that when oxygen was present derivative compounds were formed regardless of light and temperature levels.

A quote from Hidalgo *et al.* captures the essence of these extraction difficulties: "The determination of rosemary antioxidants is scarcely reproducible and may result in partial degradation" [Hidalgo *et al.* 1998]. An additional complication is that most extraction processes are time consuming. Schwarz and Ternes [1992b] commented " . . . isolation of the compounds is extremely laborious."

Growing rosemary in tissue culture provides a different set of circumstances for the researcher. To some extent, light and temperature can be controlled. Although humidity cannot be controlled, the environment of 100% humidity is very different from the open air where there is no control, and the greenhouse where there is limited control. Little research has been done to explore the production of diterpenoids in tissue-cultured rosemary and to identify the conditions, which affect the chemical profiles of the tissues and the yields of the compounds. Although

Caruso *et al.*'s recent [2000] results were reported as: "Carnosic acid in green callus and regenerated shoots of *Rosmarinus officinalis*" the details of their extraction, indicated that they extracted only regenerated shoots: "One to several grams (dry weight) of regenerating shoot primordia from callus or rooted, regenerated shoots . . ." The callus was described as "nodular" which means that shoots were beginning to emerge, so it was not a mass of undifferentiated cells.

The goals of the research for this dissertation were (1) to determine if carnosic acid and related phenolic diterpenoids are present in undifferentiated cells and regenerated plants of tissue cultured rosemary,² (2) to compare the phenolic diterpenoids present in rosemary extracts from cells and plants grown *in vitro* with those of plants grown *in terra*, (3) to produce an extract with carnosic acid as a major component, and (4) to isolate reasonable quantities of carnosic acid crystals. It was known at the outset that the achievement of these goals would depend on developing or adapting an existing protocol for the extraction of tissue cultured rosemary (particularly callus). Identification of the levels and proportions of the phenolic diterpenoids in callus and regenerated shoots would add to the growing body of research concerning the native levels of these compounds in rosemary plants. Standards of carnosic acid and carnosol have been difficult to obtain³ and though now available commercially,⁴ the prices of these valuable compounds are high enough to justify any efforts to streamline their extraction.

² This research was almost completed when Caruso *et al.* [2000] reported their results.

³ Dr. Hiroyuki Haraguchi of Fukuyama University was kind enough to provide samples of carnosic acid and carnosol.

⁴ Alexis Biochemicals in Switzerland now sells carnosic acid for \$1/mg and carnosol for \$60/mg.

MATERIALS AND METHODS

Plant Material. The following varieties of rosemary were purchased in 1994 and 1998 from the Peconic River Herb Farm, Calverton, New York: *Rosmarinus officinalis* L., *Rosmarinus officinalis* var. Hill's Hardy (a frost resistant variety), *Rosmarinus officinalis* var. Salem and *Rosmarinus officinalis* var. prostratus. The frost resistant variety was maintained outside in the research bed year round, and two of the *Rosmarinus officinalis* plants remained in the greenhouse year round along with the micropropagated plants. The other rosemary plants were moved from the greenhouse to research beds every May and returned to the greenhouse every October.

For extraction of soil-grown plants, shoots measuring 8 cm were cut from the newest growth of all of the plants except *Rosmarinus officinalis* var. Arp; flowers were removed from *Rosmarinus officinalis* and *Rosmarinus officinalis* var. prostratus. The shoots were either extracted immediately or dried at 24°C in the dark. The flowers were used immediately. For extraction of plant material grown *in vitro*, the shoots and calli were removed from the tissue culture jars, weighed, and placed in the extraction flask. All tissues were used "as is," with no grinding or chopping. Richheimer *et al.*'s report [1996] that the quantity of antioxidants produced by whole and ground rosemary were equivalent was confirmed by preliminary experiments. To avoid unnecessary exposure to oxygen, plant material was used whole.

Materials. Solvents were purchased from Fischer Scientific Company (Atlanta, GA) and from Sigma (St.Louis, MO). Water for analytical purposes was the output of a Barnstead Mega Pure System. Extracts were stored at -20°C in the dark.

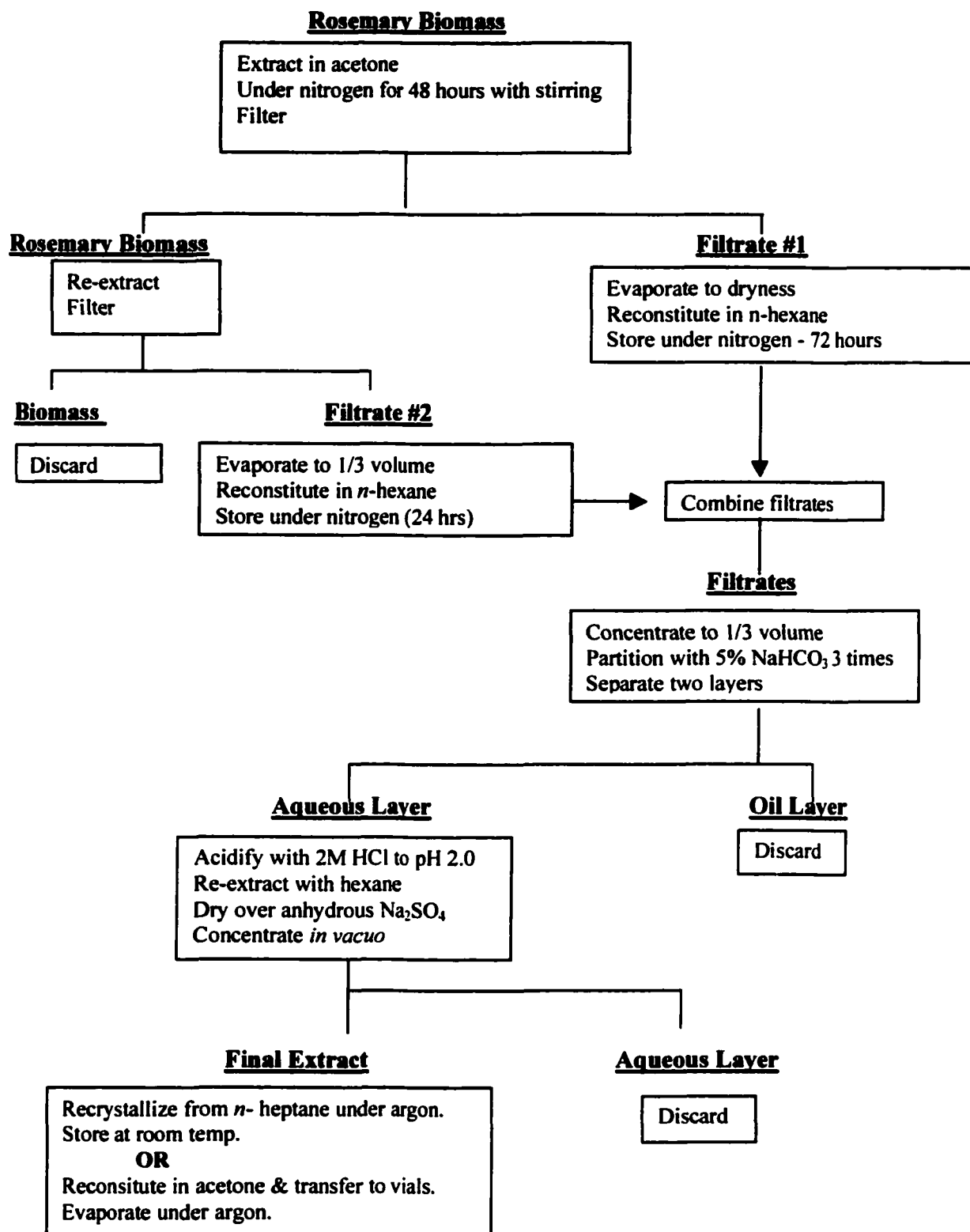
General Experimental Procedures. Melting points were measured on a Fisher Johns melting point apparatus. IR spectra were recorded as KBr discs. UV spectra were recorded on a Beckman DU 640B spectrophotometer.

Extractions. The flow chart of the primary extraction protocol is depicted in **Scheme 3.1**. Shoots, flowers, and callus from rosemary were extracted in acetone, 95% ethanol, hexane, or hexane: 95% ethanol (98:2 v/v). The biomass was introduced into a one L Erlenmeyer flask and extracted under nitrogen at 22° C on a New Brunswick gyrotory shaker at 116 rpm for 4, 24 or 48 hours. The extract was filtered under vacuum using a Buchner funnel. The residue was re-extracted with fresh solvent for twenty-four hours in the same manner as the initial extraction. The solid residue was discarded, and the filtrates from the two extractions were evaporated to one quarter of the original volume (Buchi Rotavapor-Re) at 35°C (Büchler Instruments Thermo-lift, Fort Lee, NJ), re-extracted in hexane, and stored under nitrogen for 72 and 48 hours respectively. These extracts were combined and concentrated *in vacuo* to one-third volume. The concentrated extract was then washed with an equal amount of 5% NaHCO₃ three times or until no color remained in the wash. The aqueous phase was acidified with 2 M HCl to a pH of 2.2. This phase was then re-extracted with hexane. The hexane layer was dried over anhydrous sodium sulfate and evaporated to dryness.

For HPLC analysis, the extract was reconstituted in acetone, transferred to vials, dried under argon, and stored at -20°C . For crystallization of carnosic acid, the rotary flask containing the dried extract was stored at -20°C for 24 hours. Carnosic acid crystals were produced by adding heptane to the round-bottomed flask and maintaining the flask under argon for one hour.

The suspension media were extracted in a similar manner to the solid tissues, but the first step was to extract the liquid media directly into hexane. Thereafter, the treatment was the same as for the tissues. The callus presented two problems that were resolved by (1) evaporating the extracts to one third and not to dryness and (2) repeatedly washing the hexane extract with sodium bicarbonate until no color remained instead of simply three times.

Column chromatography. Rosemary shoots were extracted in hexane: 95% ethanol (98:2 v/v) with stirring for four hours, a modification of Aeschbach and Philippossians' "Extraction, variant 2" [1993]. The extract was filtered, evaporated *in vacuo* and reconstituted in dichloromethane. A column (20 cm x 1 cm) was filled with a polyamide (Alltech, Deerfield, IL) slurry (8 g polyamide, 35 ml water), and then conditioned with 50 mL distilled water. The extract (10mL) was applied to the column. The column was eluted with 50 mL each of: 50:50 methanol: distilled water; 80:20 methanol: distilled water; 100% methanol and 99.9:1 methanol: ammonium hydroxide (NH_4OH). The last fraction was evaluated for the presence of phenolic diterpenoids using TLC, UV, and mass spectrometry.



Scheme 3.1: Diagram of extraction of phenolic diterpenoids from rosemary biomass and crystallization of carnosic acid.

TLC analysis. Extracts were tested for carnosic acid and carnosol on Polygram® silica gel plates (Sil G/UV₂₅₄) from Alltech (Deerfield, IL). The following solvent systems were tested: methanol: chloroform (18: 2); hexane: ethyl acetate (85: 15 and 70: 30); hexane: ethyl acetate: acetic acid (84.9: 15: 0.1 and 69.9: 30: 0.1). In the last solvent system (hexane: ethyl acetate: acetic acid (69.9:30: 0.1), carnosic acid and carnosol had R_f values of 0.52 and 0.47 respectively. After development, the compounds were either visualized under UV light, or sprayed with 10% sulfuric acid in ethanol and then heated at 110° C for 5 minutes. To test for antioxidants, the plates were sprayed with 0.5% phosphomolybdic acid in 95% ethanol.

HPLC analysis. The protocol was modeled on the isocratic methods of Okamura *et al.* (1994) and Richheimer *et al.* (1996). Extracts from all the samples were analyzed by high-performance liquid chromatography (HPLC) using a Waters 2690 Separations Module (Milford, MA) equipped with a 996 Photodiode Array Detector and operated with Millenium³² software. Samples, dissolved in methanol⁵, were separated on a Phenomenex Nucleosil C₁₈ column (4.60 x 250 mm, 5µm) at ambient temperature with a flow rate of 1.0 ml/min with a 65:35 mixture of acetonitrile and water with 0.5% phosphoric acid. The 996 detector scanned between 200 and 400 nm. The retention times and the concentrations were compared with those of authentic samples of carnosic acid and carnosol (gifts of Dr. Haraguchi, Fukuyama University, Japan). Once carnosic acid crystals were purified in the lab, these were

⁵ Although carnosic acid is known to degrade in methanol over a period of days, the time between dissolution and detection is less than two hours.

used as the standards. The concentrations of the other compounds were estimated against carnosic acid standards (at 228 nm) since the UV spectra of these compounds are similar (Figure 3.8).

Mass Spectrometry. Carnosic acid crystals and rosmadial crystals were analyzed by mass spectrometry (MS) using a Finnigan LCQ mass spectrometer (San Jose, CA) with a Waters 2690 Separations Module and Waters Dual λ Absorbance Detector. The instrument was equipped with an electrospray ionization source, and controlled by Xcalibur software. The capillary temperature was 230° C. Nitrogen was used as the sheath gas and helium as the auxiliary gas at flow rates of 80 and 20 units, respectively. The capillary voltage was 10V, the spray needle voltage was 4.5kV, and the tube lens offset was 0 V. A mass range of 100 to 300 amu was scanned in the negative mode.

LC-ESI MS. Callus, flower and shoot extracts were analyzed by liquid chromatography - electrospray ionization mass spectrometry (LC-ESI MS) using a Micromass Platform quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) operated in both the positive and negative ion modes (Manchester, United Kingdom) and a Hewlett Packard 1050 liquid chromatograph (Avondale, PA). The mobile phase consisted of a 65:35 mixture of acetonitrile and water with 0.15% TFA flowing at 1 mL/min that was split 80:920 μ L/min between the electrospray interface and the UV detector at 230 nm.

DPPH Assay. Shoot extracts (*R. officinalis* and *R. officinalis* var. Hill's Hardy) were dissolved in DMSO (1 mg/mL), and 50 μ L of this was mixed in a 96-well microtiter plate with 200 μ L of a 50 μ mol ethanolic solution of DPPH. The microtiter plate was incubated at 37° C for 30 minutes and absorbance was read at 515 nm. Antioxidant activity was determined as a percent inhibition by sample treatment compared with DMSO-treated controls. IC₅₀ values signify the concentration of sample necessary to scavenge 50% of DPPH free radicals. The lower the IC₅₀ value, the more potent the antioxidant.

RESULTS

Phenolic diterpenoids were identified and isolated from acetone extracts of tissue from rosemary grown *in vitro* and *in terra*. Through a pH-guided precipitation procedure, modified from Richheimer *et al.* [1996], extracts containing three major antioxidant diterpenoids, carnosic acid, carnosol and rosmanol, and a related diterpenoid, 12-methoxy carnosic acid, were obtained from callus, regenerated shoots, and shoots and flowers of cultivated rosemary. Other solvents and extraction protocols were tested, but extracting initially in acetone, re-extracting in hexane and using 5% sodium bicarbonate and acetic acid to convert the salt of carnosic acid to the acid produced extracts with clean separations of the major phenolic diterpenoids. Carnosic acid crystals, recrystallized from heptane after a cold treatment, were obtained from all the tissues containing carnosic acid. This is the first report of an extract containing major phenolic antioxidants as the principal components from green callus and micropropagated shoots of *R. officinalis*. White calli grown on Gamborg's B-5 solid media and the liquid media containing suspension cells of rosemary were found to contain no phenolic diterpenoids.

Acetone extracts from cultivated shoots and flowers, and calli of *R. officinalis*, were analyzed for the presence of carnosic acid (I) and carnosol (II) by TLC along with standard compounds, and then by LC-ESI MS. Of the three extracts analyzed by LC-ESI MS, the best separation, shown in **Figure 3.1**, was achieved with the extract from the flowers.

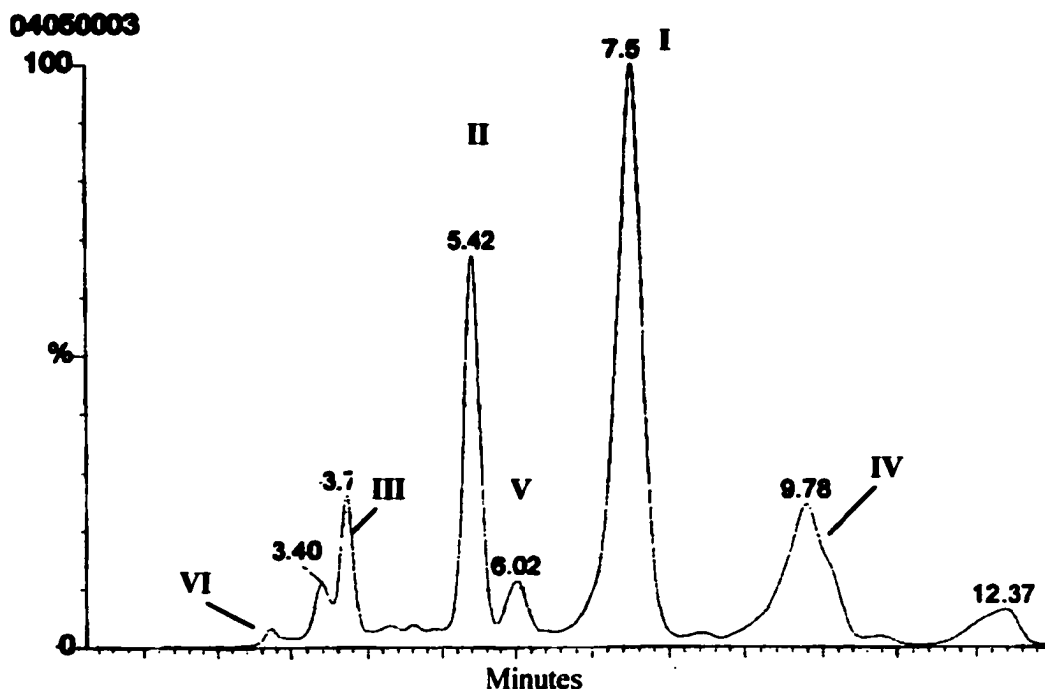


Figure 3.1 HPLC chromatogram of acetone extract from rosemary flowers, containing carnosic acid (I), carnosol (II), rosmanol (III), 12-methoxy carnosic acid (IV), rosmadial (V), and an unknown, substance 1(VI). Elution was isocratic with 65:35 acetonitrile: water with 0.5% phosphoric acid. UV detection at 230 nm.

The same extracts were analyzed by LC-ESI MS, in both the negative and positive modes, with the diode array detector set at 230 nm. **Figure 3.2** shows the mass spectra (negative mode) of five of the six compounds in the flower extract, carnosic acid (I), carnosol (II), 12-methoxy carnosic acid (III), rosmanol (IV), and rosmadial (V). There was one unknown (VI), with a mass of 306. In the positive mode, the most abundant compound was also carnosic acid, but the second most abundant was the sodium adduct of carnosol, with a mass of 353 (**Figure 3.3**), rather than carnosol. Carnosic acid ionizes more readily in the negative mode so this mode was used in later analyses.

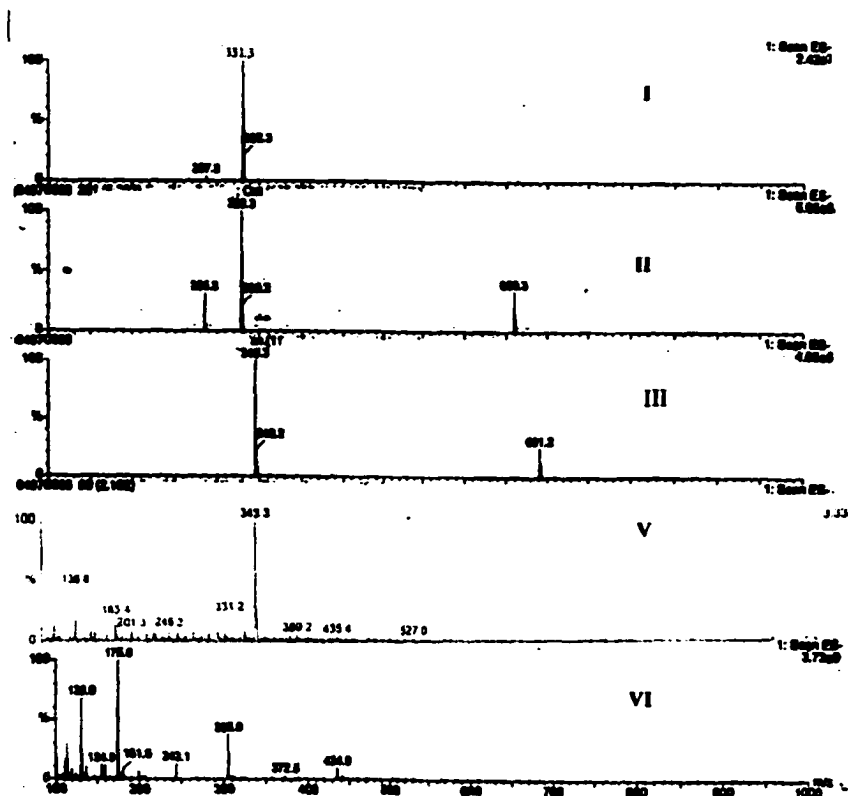


FIGURE 3.2 ESI MS (negative ionization) of five of the six compounds in the acetone extract of flowers of *Rosmarinus officinalis*, in order of abundance: carnosic acid (I), carnosol (II), rosmanol (III), rosmadial (V), and substance 1 (VI). 12-methoxy carnosic acid (IV) the third most abundant, is not shown

The relative abundance of carnosic acid compared with the other components of the extract is confirmed by the total ion counts (Figure 3.4). The masses for the known compounds match those reported in the literature (Table 3.1).

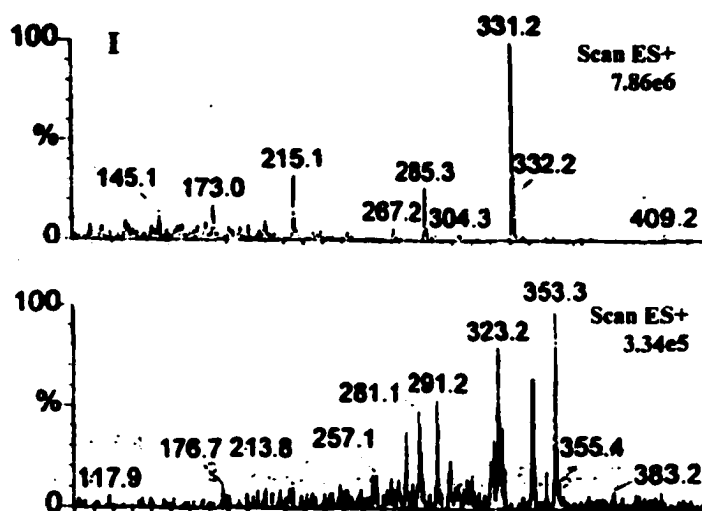


Figure 3.3 LC-ESI MS (positive ionization) mass spectra of the two most abundant compounds in an acetone extract of flowers of *Rosmarinus officinalis*, in order of abundance: carnosic acid (I) and the sodium adduct of carnosol (m/z 353).

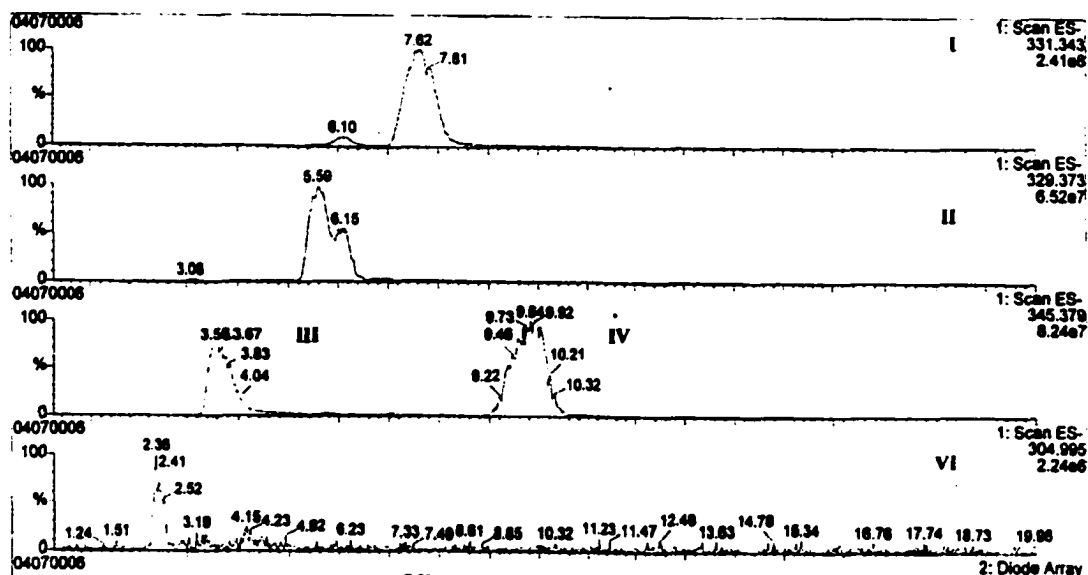


Figure 3.4 Total ion counts of five of the six compounds in an acetone extract of flowers of *Rosmarinus officinalis*, analyzed by LC-ESI MS in the negative mode, in order of abundance: carnosic acid (I), carnosol (II), 12-methoxy carnosic acid (III), rosmanol (III), 12-methoxy carnosic acid (IV), substance 1 (VI). Rosmadial (V) is not shown.

Carnosic Acid	Carnosol	Rosmanol	Rosmadial
	[Wu <i>et al.</i> 1982]		
	[Dimayuga <i>et al.</i> 1991]		
[Schwarz and Ternes 1992 II]			
[Ho <i>et al.</i> 1994] ⁶	[Ho <i>et al.</i> 1994] ⁷		
[Cuvelier <i>et al.</i> 1994]	[Cuvelier <i>et al.</i> 1994]	[Cuvelier <i>et al.</i> 1994]	[Cuvelier <i>et al.</i> 1994]
[Bicchi <i>et al.</i> 2000] ⁸	[Bicchi <i>et al.</i> 2000] ⁹		

Table 3.1 Literature references for mass spectral data on four antioxidant phenolic diterpenoids identified in acetone extracts of rosemary tissues.

Rosmanol (III) and 12-methoxy carnosic acid (IV) are isobaric, each with a molecular weight of 346 (**Figure 3.5**), but rosmanol, the more polar compound, eluted first (**Figures 3.1 and 3.4**).

⁶ HPLC/PB/MS

⁷ HPLC/PB/MS

⁸ HPLC/ELSD/MS and HPLC/PB/MS

⁹ HPLC/ELSD/MS and HPLC/PB/MS

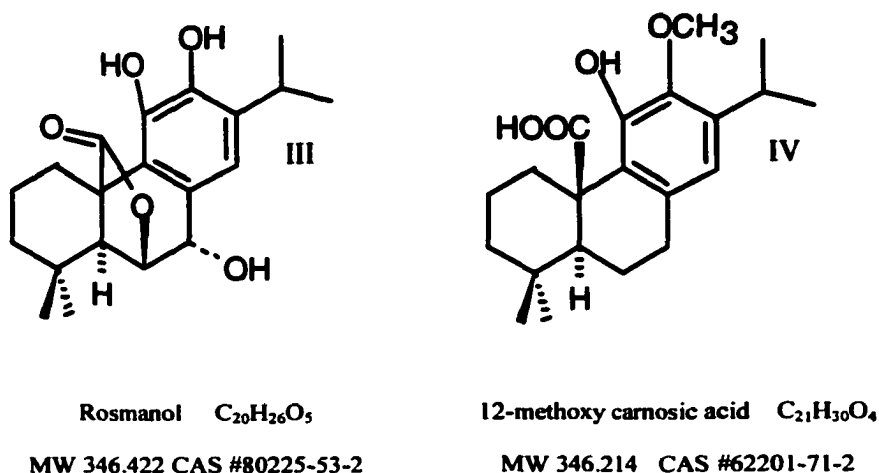


Figure 3.5 The structures, molecular formulae, and molecular weights of rosmanol (IV) and 12-methoxy carnosic acid (III). The molecular weights are the same, but based on polarity these two compounds eluted at different times

In a subsequent set of experiments, four extracts from each type of tissue were analyzed by HPLC equipped with a photodiode array detector, in which the lower limit of detection for carnosic acid was determined to be 10 ng. The isocratic solvent systems described by Okamura *et al.* [1994] and Richheimer *et al.* [1996], combining acetonitrile mixed with water containing 0.5% phosphoric acid in a 65:35 ratio, resulted in a good separation of the terpenoids. The following tissues were analyzed: callus and micropropagated shoots grown on MS media with 4 mg/L BAP, 10 mg/L IAA and 1 mg/L kinetin, callus on MS media with varying concentrations of 2,4-D and BAP, cultivated shoots (fresh and dried) and flowers, callus grown on Gamborg's B-5 media and suspension media. The white callus (Gamborg's B-5) and the liquid suspension media

showed the presence of only one compound in common with the other extracts, the early eluting unknown, substance 1 (VI). Seven out of the eight surviving sets of calli grown on MS media with BAP and 2,4-D (Chapter Two, **Table 2.2**) produced very complex extracts with no detectable levels of phenolic diterpenoids. The extract with the highest level of BAP (11.25 mg/L) and the second highest level of 2,4-D (0.11 mg/L) was the only one of the eight sets of calli from the multifactorial¹⁰ experiments to have detectable levels of carnosic acid and carnosol (**Table 3.3**).

Of the extracts that contained phenolic diterpenoids as major components, each type of tissue contained at least carnosic acid and carnosol, along with the early eluting unknown, substance 1 (VI). The carnosic acid standard eluted at 12.5 minutes (**Figure 3.6**); its absorbance spectrum showed maxima at λ 206 and 284 nm with a shoulder at 228 nm (**Figure 3.8**). The carnosol standard eluted at 8.6 minutes; its absorbance spectrum showed maxima at λ 206 and 284 nm (**Figure 3.8**). The retention times and spectra of the other compounds (rosmanol, rosmadial and 12-methoxy carnosic acid) were compared with those from the previous LC-ESI MS analyses and literature data [Inatani *et al.* 1982; Nakatani and Inatani 1984; Al-Hazimi 1986; Djarmati *et al.* 1993; Cuvelier *et al.* 1996].

Carnosic acid was the predominant compound in all of the extracts, except the callus extracts and one of the regenerated shoot extracts. The first callus and the first

¹⁰ As described in Chapter Two, there were originally sixteen sample sets, but the calli grown on the two higher levels of 2,4-D (0.55 and 1.10 mg/L) died within six weeks of inoculation.

regenerated shoot extracts produced almost equal amounts of carnosic acid and carnosol; they were also the only extracts which produced the full complement of related compounds, i.e. rosmanol, rosmadial, 12-methoxy carnosic acid and substance 1. These results may reflect an extraction learning curve and resulting degradation, rather than the inherent chemical composition of the tissue. In the regenerated shoot extracts, carnosic acid dominated, carnosol or substance 1 was the second most abundant, and 12-methoxy carnosic acid was present at low levels. Rosmadial was detectable in two of the extracts (Figure 3.7, samples B and C). In the callus extracts from tissue grown on MS with IAA as the auxin, carnosol was the dominant compound in three out of six extracts.

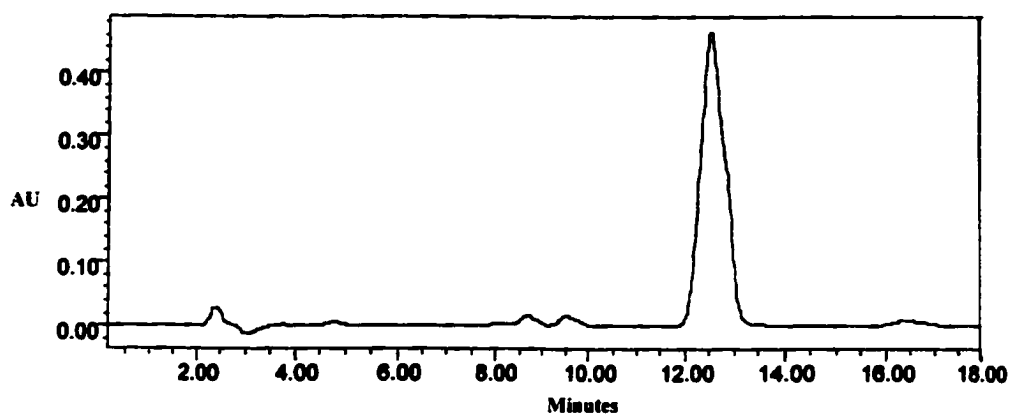


Figure 3.6 HPLC chromatogram of carnosic acid standard purchased from Alexis Biochemicals. Column Phenomenex Nucleosil C₁₈ column 4.60 x 250 mm, 5 μ m; isocratic: 65:35 acetonitrile: water with 0.5% phosphoric acid; flow rate 1 mL/min; UV detection at 228 nm.

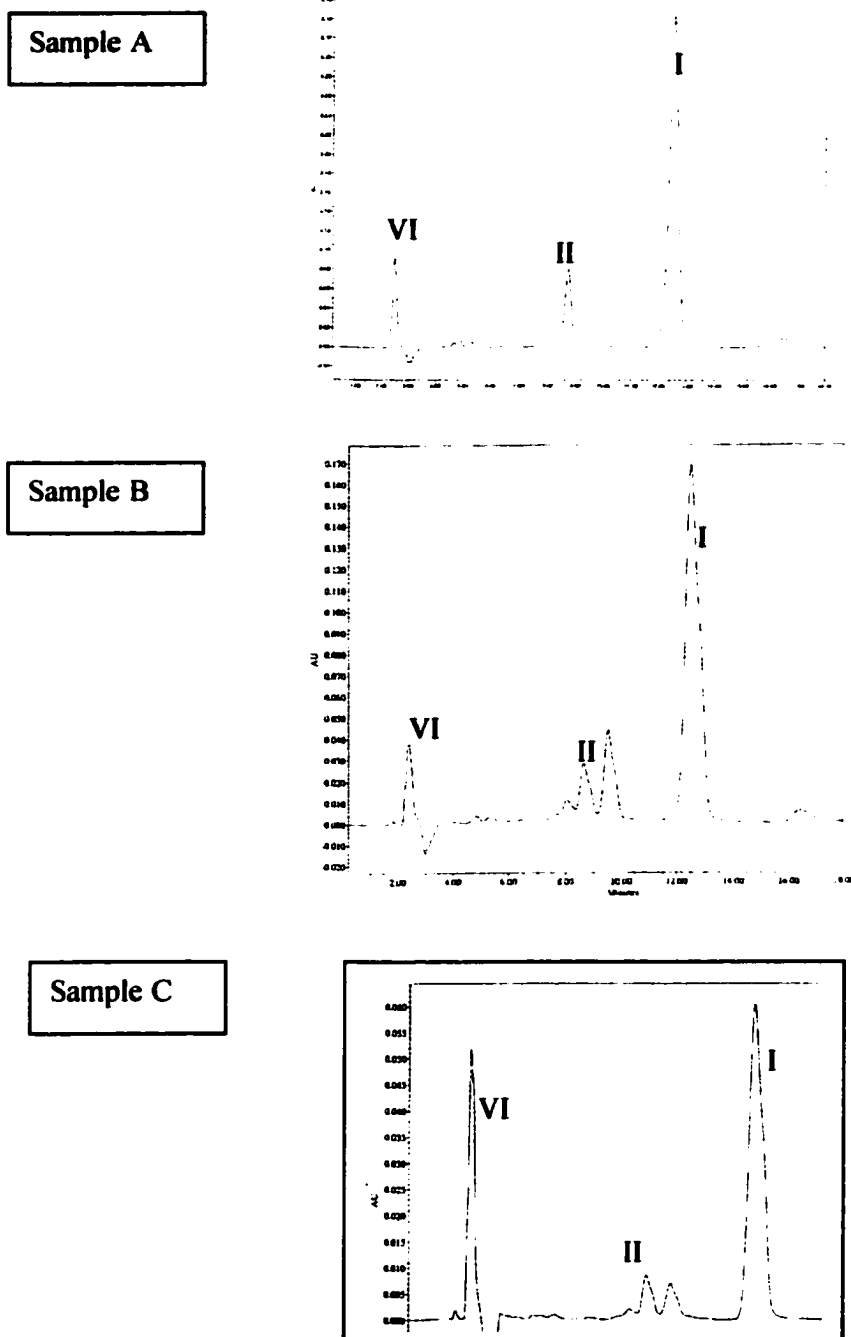
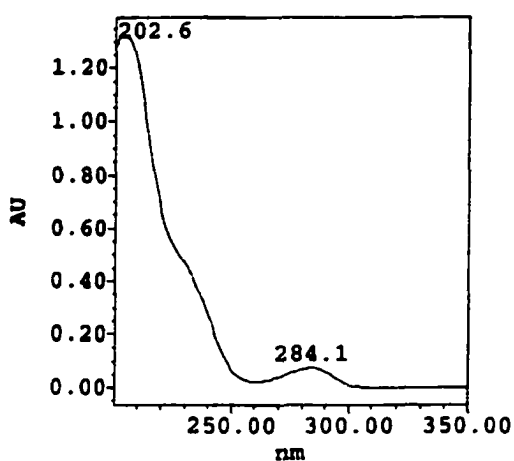
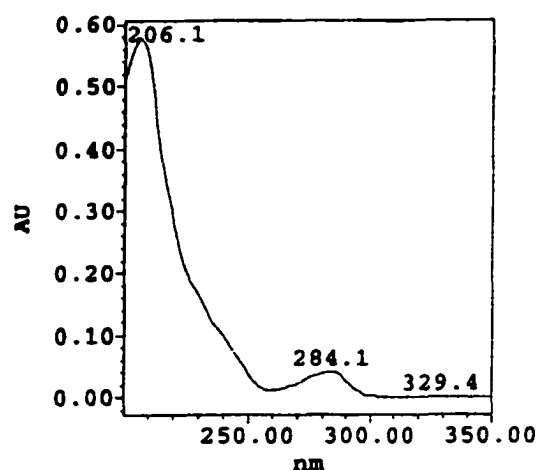


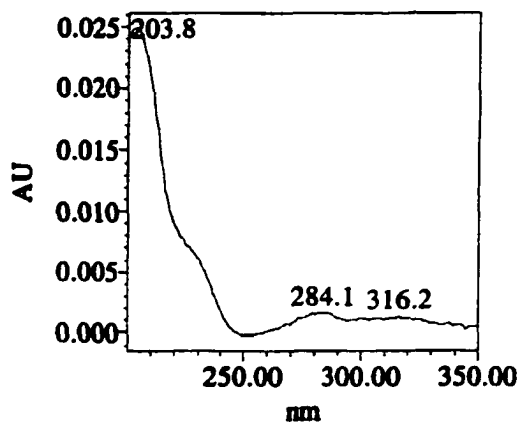
Figure 3.7 HPLC chromatograms of acetone extracts of from three different samples of regenerated shoots of *Rosmarinus officinalis*. Phenomenex Nucleosil C₁₈ column 4.60 x 250 mm, 5 μ m; isocratic: 65:35 acetonitrile: water with 0.5% phosphoric acid; flow rate 1 mL/min; UV detection at 228 nm.



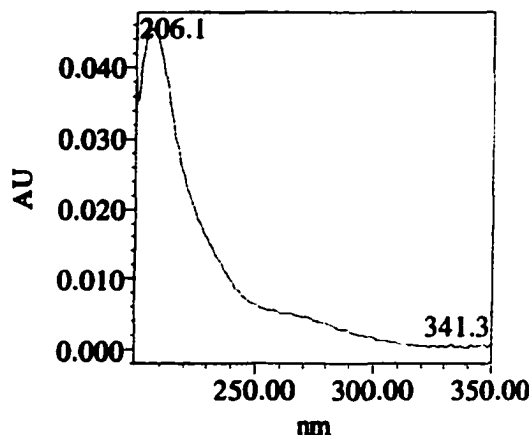
UV spectrum of carnosic acid (I)
from regenerated shoots



UV spectrum of carnosol (II)
from regenerated shoots



UV spectrum of 12-methoxy
carnosic acid (IV) from callus



UV spectrum of unknown
from callus

FIGURE 3.8 UV spectra (detected at 228 nm) of three major phenolic diterpenoids (carnosic acid I, carnosol II, and 12-methoxy carnosic acid (III) identified in acetone extracts of rosemary tissue grown *in vitro*. Note the shoulder at 228 nm, which is typical of the carnosic acid spectrum. The unknown has a relative retention time 1.1 times that of carnosol and appears in all of the tissue culture extracts. Phenomenex Nucleosil C₁₈ column 4.60 x 250 mm, 5 μ m; isocratic: 65:35 acetonitrile: water with 0.5% phosphoric acid; flow rate 1 mL/min. UV detection at 228 nm.

In the extracts from tissue grown *in vitro*, the percentage of carnosic acid ranged from 0.01% to 0.26% of the starting biomass. The amount of carnosol was less than 0.03%. The low yields of carnosic acid and carnosol in the callus and the regenerated shoots were a combination of low net yield of phenolic diterpenoids from the starting material and low content of carnosic acid in the extracts. The average yield of phenolic diterpenoids from callus extracts was 0.56% (n=6) and for micropropagated shoot extracts it was 0.87% (n=7). These figures are substantially lower than the mean of 1.21% for the yield of phenolic diterpenoids in the extracts from fresh shoots and the mean of 1.62% for the yields from dried shoots. In the extracts evaluated by HPLC, the percentage of carnosic acid in the extracts from the fresh and dried shoots ranged from 62% to 95%, while in the regenerated shoots it was below 71% and for callus extracts it was below 15%. The concentration of each phenolic diterpenoid in the extract was estimated using a concentration curve based on carnosic acid standards since the UV spectra of these compounds are similar. Detection was at 228 nm. The net result of combining the yields (%) with the carnosic acid content (%) was that the lowest yield of carnosic acid from the fresh shoots was 0.75%, whereas the lowest yield for the callus was 0.01%. These figures were calculated by multiplying the % yield of the extract times the % that each compound represented of the extract, divided by 100:

Fresh shoots: 0.75% carnosic acid content = net yield of 1.21% (weight of extract / weight of initial biomass x 100) multiplied by 62% carnosic acid content in the extract (peak area / total area x 100) divided by 100.

Callus: $0.01\% = \text{net yield of } 0.56\% \left(\frac{\text{weight of extract}}{\text{weight of initial biomass}} \times 100 \right) \text{ multiplied by } 2\% \text{ carnosic acid content in the extract } \left(\frac{\text{peak area}}{\text{total area}} \times 100 \right) \text{ divided by } 100.$

The range of yields of carnosic acid for all the tissues are shown in **Table 3.2**.

Tissue Source	Range of Carnosic Acid
Cultivated Shoots (fresh)	1.3 – 2.0%
Regenerated Shoots	0.7 – 1.05%
Callus (MS/IAA and BAP)	<0.5%
Callus (B5)	0%
Liquid Media (MS)	0%

Table 3.2 Ranges of carnosic acid content of different tissues, expressed as percentages, calculated by multiplying the yield of the extract times the percentage which carnosic acid represented of the overall yield.

There was also great variability in the concentration of each compound from one extract of *in vitro* grown tissue to another. The HPLC chromatograms in **Figure 3.7** are examples of extracts from three samples (A,B,C) of regenerated shoots. For comparison, **Figure 3.6** is an HPLC trace of carnosic acid powder purchased from Alexis Biochemicals. In the regenerated shoot extracts, the carnosol to carnosic acid ratio ranged from 17% to 70%, with a mean of 41% (standard error of 11.6%), calculated by comparing the areas under the peaks. In the extracts of cultivated shoots, this ratio (carnosol to carnosic acid) was below 10% and in flowers the ratio ranged from 5% to 15%. Rosmadial, a phenolic diterpene lactone, was present in several extracts. Rosmanol was either absent or present only in trace amounts in all of the extracts analyzed by HPLC.

Calli from three different sets of experiments were extracted in acetone. As described above, green callus was grown on MS media with IAA as the auxin and 4 mg/L of BAP as the cytokinin. These calli were extracted six to eight months after inoculation and displayed the full range of phenolic diterpenoids. In another experiment, B5 was used as the basal medium since previous experiments had shown that white callus was produced consistently on this basal medium. The HPLC chromatogram of the extract of white callus identified only one compound, the early eluting substance 1, in common with the extracts from cultivated shoots. The fact that carnosic acid was not present in this tissue is consistent with the recent discovery by Munné-Bosch *et al.* (2001) that carnosic acid is synthesized in the chloroplasts and oxidizes to isorosmanol in these organelles. Carnosol and rosmanol were also located in the chloroplasts according to this recent study.

The third experiment using callus was based on determining if particular hormonal additions and concentrations affected diterpenoid production in callus tissue. Sixteen different media formulations were prepared with four different concentrations of BAP (0.225 mg/L to 11.25 mg/L) and four different concentrations of 2,4-D (0.055 mg/L to 1.10 mg/L) (Table 2.2, Chapter 2). 2,4-D was substituted for IAA since it has been observed that green callus predominates with this auxin. The eight sets of calli grown on MS media with the two higher concentrations of 2,4-D (0.55 and 1.1 mg/L) were weak and developed necrosis within six weeks of inoculation (Table 3.3). With one exception, the extracts of the eight remaining sets of calli with the two lower concentrations of 2,4-D (0.055 and 0.110 mg/L) contained small quantities of numerous compounds with no prevalence of phenolic

diterpenoids. The calli grown on MS media with 0.11 mg/L 2,4-D and 11.25 mg/L BAP produced detectable levels of carnosic acid and carnosol (**Table 3.3**)

BAP 2,4 -D	0.225 mg/L	1.125 mg/L	2.250 mg/L	11.250mg/L
0.055 mg/L	1	1	1	1
0.110 mg/L	1	1	1	Carnosic Acid Carnosol
0.550 mg/L	2	2	2	2
1.100 mg/L	2	2	2	2

Table 3.3 Results of TLC and HPLC extracts from multifactorial experiment.

TLC. 65:35 hexane: ethyl acetate.

HPLC. Phenomenex Nucleosil C₁₈ column 4.60 x 250 mm, 5 μ m; isocratic: 65:35 acetonitrile: water with 0.5% phosphoric acid; flow rate 1 mL/min. UV detection at 228 nm.

Note 1: No detectable levels of either carnosic acid or carnosol.

Note 2: Calli browned and died at an early stage.

Both dried and fresh shoots were analyzed and the only difference was that the former contained almost exclusively carnosic acid and carnosol, indicating minimal degradation during the extraction process. The fresh shoot and flower extracts were comparable in content and proportions, as analyzed by HPLC with detection at 228 nm.

Carnosic acid was isolated and crystallized from the acetone extracts of callus and micropropagated shoots, and from dried and fresh shoots and flowers of cultivated *Rosmarinus officinalis*. The identification of these golden prisms, recrystallized using heptane, from fresh shoots and callus was confirmed by mass spectrometry (**Figure 3.9**), TLC compared with carnosic acid standard, HPLC

relative retention times (**Figures 3.6 and 3.11**), and literature values for melting point (**Table 3.4**), UV spectrum (**Figure 3.8**), and IR spectrum (**Figure 3.10**) compared with the standard of carnosic acid provided by Dr. Haraguchi.

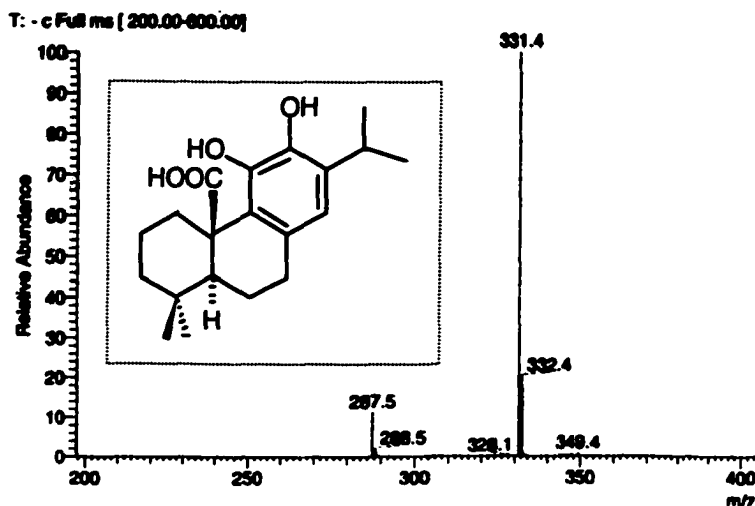


Figure 3.9 Direct injection ESI MS (negative ion) for carnosic acid crystals isolated from *Rosmarinus officinalis* callus grown on MS media with BAP.

Melting point data for the crystals matched those of the original literature citation, 185-190° C with decomposition, recorded by Wenkert *et al.* in 1965, and subsequently cited in most references as the standard [Paris *et al.* 1993; Haraguchi *et al.* 1995]. Slight departures from this reference standard have been reported by several research groups (**Table 3.4**).

Literature reference	Crystal description	Solvent for recrystallization	Melting point
[Aeschbach and Philippossian 1993]	Colorless	Hexane	193-199° C
[Masuda <i>et al.</i> 2001]	Pale yellow needles	Water/acetic acid	194-195°C
[Paris <i>et al.</i> 1993]	Pale yellow prisms	Hexane	189-193°C
[Richheimer <i>et al.</i> 1996]	Off-white	Heptane	190° C
[Wenkert <i>et al.</i> 1965]	Colorless	Hexane	185-190° dec.

Table 3.4 Melting points for carnosic acid crystals and recrystallization solvent as reported in the literature.

The differences in **Table 3.4** reflect the purity of the crystals. Masuda *et al.* [2001] isolated carnosic acid using Richheimer's protocol and then purified it using preparative HPLC so their melting point reflects a higher degree of purity than other researchers who recrystallized to purify.

The original carnosic acid standard and golden prisms isolated from *Rosmarinus officinalis* var. Hill's Hardy were analyzed by infrared spectroscopy (**Figure 3.10**). The peak at 3530 cm^{-1} represents the phenolic hydroxyls, the sharp peak at 1650 cm^{-1} is characteristic of the carboxyl group, and the stretch at 2950 cm^{-1} represents the aromatic C-H stretch. These spectra are very similar to methyl carnosate [Cuvelier 1994], which has the same di-orthophenolic functions and an isopropyl group on the adjacent carbon¹¹, but which has substantially less biological activity. According to data from [Vasiliev *et al.* 1986], the difference between 1650 to 1700 can represent the difference in the antioxidative power of two compounds. They found that the shift from the range $1600\text{--}1700\text{ cm}^{-1}$ to $1700\text{--}1800\text{ cm}^{-1}$ that occurred when carnosic acid was stored for six months corresponded to a decline in the antimicrobial activity towards *Staphylococcus aureus*. They found that the

¹¹ Methyl carnosate has absorptions at 3500, 2950, and 1700 [Cuvelier *et al.* 1994].

intramolecular hydrogen bonding between the carbonyl at carbon 10 and the hydroxyl group at carbon 11 contributed to the antioxidative power of carnosic acid. Wenkert *et al.* (1965) reported a Nujol analysis (OH 2.88m, sharp and 2.99 m, broad; C=O 6.06 u). Schwarz and Ternes (1992b) reported peaks at 1645 and 3540 (Nujol), but did not publish a spectrum.

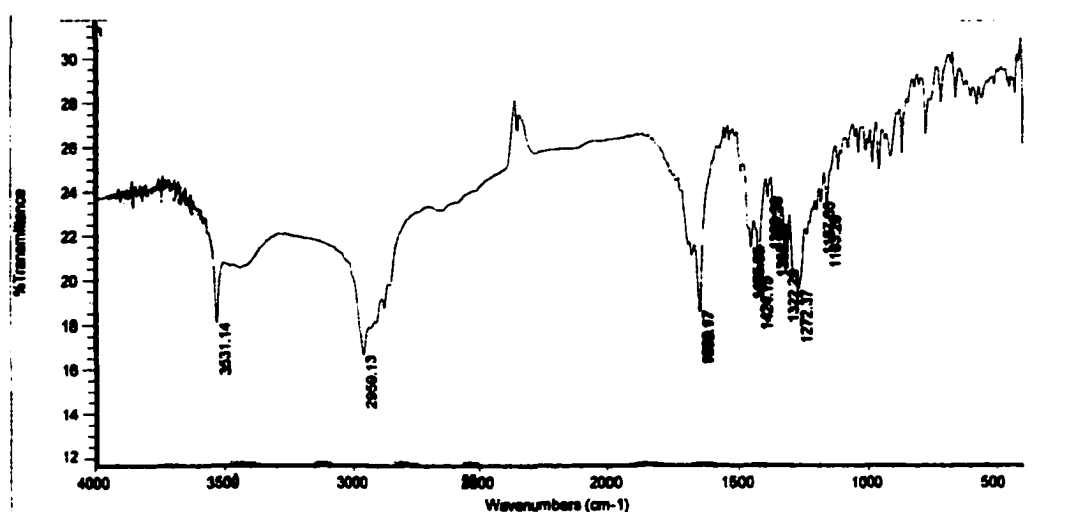


Figure 3.10 IR spectrum [KBr pellet] of crystals isolated from acetone extract of shoots from *Rosmarinus officinalis* var. Hill's Hardy.

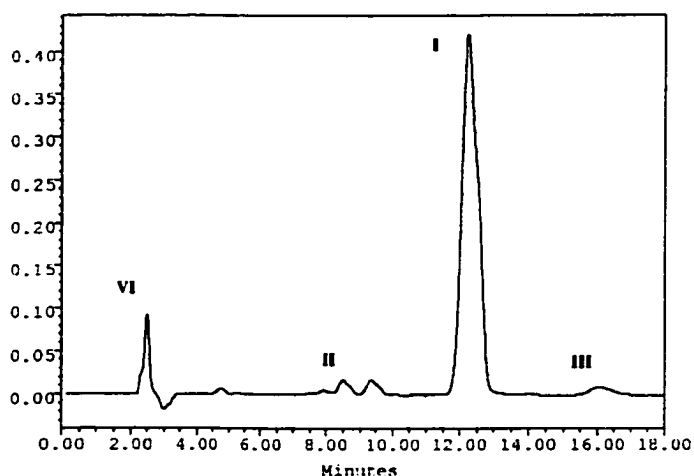


Figure 3.11 HPLC spectrum of carnosic acid crystals, recrystallized from heptane, isolated from acetone extract of shoots of *R. officinalis* var. Hill's Hardy. Column Phenomenex Nucleosil C₁₈ column 4.60 x 250 mm, 5 μ m; isocratic: 65:35 acetonitrile: water with 0.5% phosphoric acid; flow rate 1 mL/min. UV detection at 228 nm.

Two shoot extracts were evaluated for antioxidant activity using the DPPH assay. Both showed high antioxidant activity, 12.37 and 14.22 μ g/mL (IC₅₀) for *R. officinalis* and *R. officinalis* var. Hill's Hardy respectively.

When procedures other than pH-guided extraction of phenolic diterpenoids were used, the chemical profiles of the extracts were different. For example, a 95% ethanol extract of fresh shoots from *R. officinalis* var. Salem, analyzed by LC/ESI-MS, identified carnosol as the sole compound. Since Richheimer *et al.* [1996] reported success in obtaining carnosic acid from ethanol extracts, the reason for carnosol alone was most likely the length of time spent extracting the tissue. This was one of the first extractions so it took longer than later extractions. When a modification of Nestlé's column chromatography protocol [Aeschbach and Philippossian 1993] was utilized, rosmadial, a diterpenoid lactone from rosemary, was the only compound with antioxidant properties. Rosmadial was identified by ESI-MS (**Figure 3.12**).

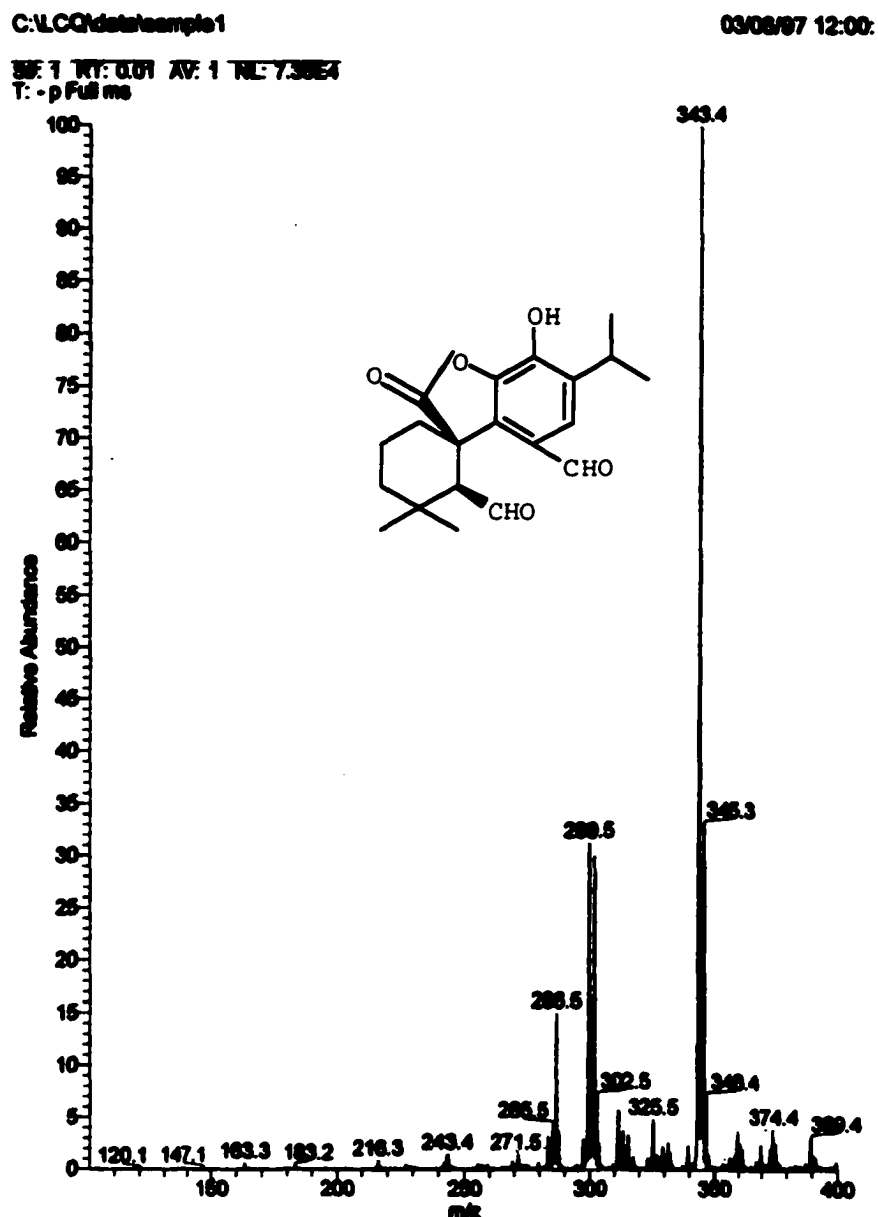


Figure 3.12 Direct injection ESI MS (negative ion) of rosmadial, extracted from shoots of *Rosmarinus officinalis* and purified by column chromatography.

DISCUSSION

The extraction protocol, developed by Paris *et al.* [1993] and modified by Richheimer *et al.* [1996], was further refined by selecting acetone as the initial extraction solvent, testing the duration of the extractions, altering the ratios of solvent to tissue, and developing ways to maintain samples in an atmosphere of inert gas during the stages of extraction. Additionally, the procedure was adapted to the particular requirements of undifferentiated cells obtained from tissue culture and also to the liquid media containing suspension cells. The callus tissue, for example, contained significantly larger amounts of oil than shoots or flowers, so exhaustive washing with sodium bicarbonate was required. Paris' group extracted initially in hexane under nitrogen and then proceeded directly to wash the hexane with sodium bicarbonate. The subsequent steps were the same as outlined in **Scheme 3.1**. Richheimer's group extracted initially in ethanol, evaporated the extract to dryness, and then proceeded as in **Scheme 3.1**. The two major changes in this protocol adopted in this lab were to extract initially in acetone and to evaporate that extract *in vacuo* down only to one third of the original volume rather than to dryness.

Rosemary shoots were also extracted in 95% ethanol and hexane: ethanol (98:2 v/v), but preliminary analysis identified either too many compounds (hexane: ethanol extract) or too few (ethanol extract). An early experiment extracting initially in ethanol produced an extract consisting solely of carnosol. Since carnosol was not the desired product, an initial extraction with ethanol was not repeated. However,

given the price of carnosol (\$60/mg), recently introduced to the market, this particular experiment should be repeated.

The fact that acetone was an effective initial extraction solvent is supported by observations of several researchers. The Hauser patent [Anonymous 1995] stated that 90% of the carnosic acid could be extracted with acetone, with purity between 20% and 30%. Hidalgo's group [1998] compared organic solvent extraction using acetone with supercritical fluid extraction, commenting that only "exhaustive extraction" with acetone provided results comparable with SC- CO₂ extraction. Similarly, Tena *et al.* [1993] found that the recovery rate with acetone was 73% of that from supercritical fluid extraction.

The procedure for the crystallization of carnosic acid was also refined to include the use of argon for drying down and the introduction of low temperature (20°C) at the penultimate step. Crystallization is not a predictable process and the literature contained little guidance. For example, the Hauser patent advised: "That solution is chilled to produce crystalline carnosic acid...Other crystallization procedures may be employed within the knowledge of one skilled in the art." [Anonymous 1996].

Other issues concerning extraction techniques and material preparation included: fresh versus dry, ground vs. whole. Since the experiments were based on comparisons between tissues originating in the tissue culture environment and the cultivated environment, fresh tissue was used to provide the most comparable results. The decision not to use dried material as the main tissue from the cultivated plants

was also based on temperature concerns.¹² This decision was later confirmed by the conclusions of Ibanez's group that "rosemary leaves treated at the mildest thermal conditions provided a higher antioxidant activity than those dried at higher temperatures" [Ibanez *et al.* 1999]. It has been established that carnosic acid is unstable at temperatures over 120° C. Tena *et al.* [1997] found that even using liquid nitrogen for grinding and freezing reduced the carnosic acid yield. Since acetone disrupts membranes effectively, and the exposure to air had to be minimized, the decision was made to use all plant material "as is," with no chopping, grinding, or pulverizing.

The yields of the phenolic diterpenoids in tissue from cultivated rosemary were in line with the results of other researchers, particularly those of Munné-Bosch's group which has performed the most recent, most thorough evaluation of carnosic acid content in cultivated rosemary [Munné-Bosch *et al.* 2001]. The data on the identification and yields of phenolic diterpenoids in the tissue grown *in vitro* are being reported for the first time. Though Caruso *et al.* [2000] reported the presence of carnosic acid in regenerated shoots of rosemary, this was the only phenolic diterpenoid identified and no yield data was provided. The yields from the callus tissue were low, but in line with reported results for diterpenoid production in callus of members of the Labiatae family [Banthorpe *et al.* 1990; Knoss 1995].

The final extracts of the callus and shoots grown *in vitro* also invariably contained a white powder which was soluble only in water. Although, this substance was easy to isolate from the acetone extract, it may have interfered with the extraction

¹² As noted in the Materials and Methods section, dried shoots were extracted for comparison with fresh shoots, but these were dried at 24° C.

process and permitted more degradation of carnosic acid than occurred with the other tissues. As discussed in Chapter One, most researchers now agree that carnosol is a degradation product which results from exposure of carnosic acid to air during the extraction process.

CHAPTER FOUR

Conclusions and Directions for Future Research

The primary purpose of this research was to determine if the major antioxidant diterpenoids, known to exist in rosemary grown *in terra*, were present in rosemary tissue grown *in vitro*. Given the importance of carnosic acid and carnosol for human health, the high cost of these compounds, and the extreme variability of their concentration in cultivated and wild rosemary, it seemed that tissue culture should be examined as a potential source of consistent yields of these compounds.

Carnosic acid, carnosol, rosmanol and 12 methoxy carnosic acid are present in green callus and shoots regenerated from green callus of rosemary. The chemical profiles of the extracts from these tissues are similar to those of the cultivated shoots and flowers, but the yields are lower. As Robert Aeschbach [Aeschbach and Philippossian 1993] stated, carnosic acid can be extracted only from “a preparation of vegetable material already enriched with carnosic acid,” so there is no question that carnosic acid is present in tissue cultured plant material. The “naturalness” of the other three compounds continues to be a question. Many research groups, including those of Marie-Elisabeth Cuvelier, Karin Schwarz and Robert Aeschbach, believe that carnosol, rosmanol and 12-methoxy carnosic acid are degradation products of carnosic acid, resulting from the exposure to oxygen or the use of methanol in the extraction process. Aeschbach stated “. . .it can be incidentally demonstrated that the freshly cut leaves of rosemary do not contain carnosol.” [Aeschbach and Philippossian 1993]. Munné-Bosch *et al.* also considered these compounds to be

derivatives, but concluded that the degradation occurs in the plant as carnosic acid oxidizes to protect the plant from high temperature and illumination. The truth is yet to be determined.

Certainly, in this series of experiments, where the same extraction protocol was applied to all tissues, the results are different for the different tissues. Just as Munné-Bosch's group found that carnosic acid levels varied with the season and Richheimer *et al.* determined that the carnosol/carnosic acid ratio varied by geographic origin, these results show that carnosic acid levels and the carnosol / carnosic acid ratio may vary with the source of the tissue. The chemical profile of the regenerated shoot extracts was similar to those of the extracts of cultivated shoots and flowers. The fact that carnosol and carnosic acid were present in callus tissue in almost equal amounts may reflect the much higher water content of undifferentiated cells compared with differentiated cells. Alternatively, the ratio could reflect a different set of stresses in tissue grown *in vitro*. According to Munné-Bosch's group carnosic acid degrades to carnosol *in vivo* under stress and both are present in the chloroplasts. Although humidity is high and should not be a source of stress, the light and temperature levels of the growth chamber are different from those in the field and could be at stressful levels. The effects of different sources of stress as causes for these proportions should be further examined. Light, temperature and photoperiod can all be easily altered in the tissue culture system to determine if one factor is more potent in influencing the levels of carnosic acid and carnosol in callus cells and regenerated plants of rosemary. Using tissue culture may ultimately contribute to an

understanding of the levels of these antioxidant compounds in rosemary that in turn could result in higher, more consistent yields.

The overall result that callus tissue contains substantially lower levels of carnosic acid and related diterpenoids is not surprising based on reports in the literature of the production of diterpenoids in tissue culture, as discussed in Chapters One and Three. At the outset of these experiments, serious consideration was given to using elicitors to enhance the yields of carnosic acid and carnosol in the callus and media of rosemary, however, the yields are so low and the procedures are so labor intensive that it would seem other means should be pursued. It would be worthwhile, for example, to adapt one of the rapid extraction protocols for immediate injection into the HPLC to determine if in fact the low yields can be attributed to the lengthy extraction process or to inherently low yields.

Research concerning the growth of rosemary using the tissue culture systems developed in these experiments should be pursued. The light and temperature studies revealed that growth of rosemary in tissue culture was significantly affected by levels of illumination and heat. The Munné-Bosch group in Spain has recently made some interesting discoveries about the variability in levels of carnosic acid as a function of light, temperature and water availability. Tissue culture provides an excellent research system to examine the production of carnosic acid and carnosol at varying light and temperature levels, with a constant level of humidity.

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