ENZYMATIC SYNTHESIS AND IMMUNOSUPPRESSIVE ACTIVITY OF NOVEL DESMETHYLATED IMMUNOMYCINS (ASCOMYCINS)†

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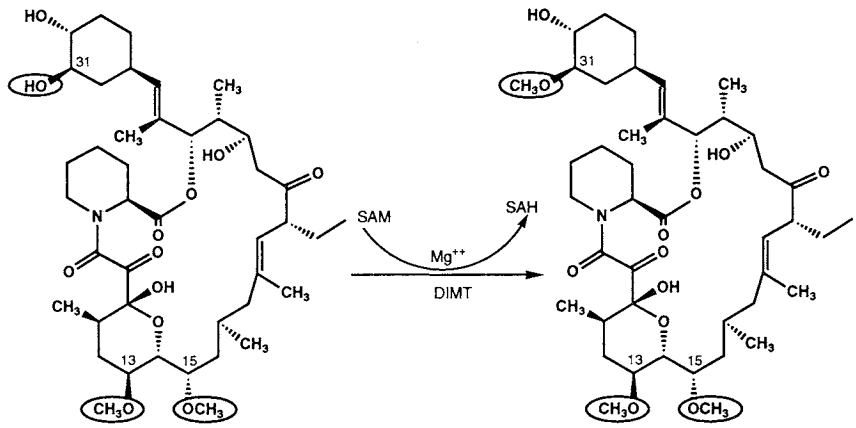
31-*O*-Desmethylimmunomycin O: methyltransferase (DIMT), an enzyme involved in the biosynthesis of immunomycin (ascomycin/FR-900520), was used to synthesize three analogs of this immunosuppressant compound. These compounds were assigned the following structures: 13-*O*-desmethyl-, 15-*O*-desmethyl- and 13,15-*O*-bisdesmethyl-immunomycins. Two of these compounds, namely, 15-*O*-desmethyl- and 13,15-*O*-bismethyl-immunomycins have novel structures and were examined for possible immunosuppressive activity by *in vitro* T-cell proliferation assay. The results showed that methylation of the C-15 hydroxyl is critical for full biological activity of the immunomycin.

Cyclosporin A[1] and FK-506[2] are two natural products with potent immunosuppressive properties which are being used as therapeutic agents for the treatment of organ transplant rejection[3]. In addition, these compounds have been increasingly utilized as research tools to investigate the mechanisms of lymphocyte activation[4]. Because of these potential applications, the search for new molecules with similar activity has intensified in recent years. As a result of such efforts, an immunosuppressant agent, designated as immunomycin, was isolated from the broth of *Streptomyces hygroscopicus* var. *ascomyceticus* ATCC 55087[5]. This compound was later proved to be identical to the previously reported antifungal agent, ascomycin[6] or FR-900520[7] and represents the C-21 ethyl analog of FK-506.

Modified immunomycin structures are of particular interest as they may possess unique pharmacological properties and also should help to define the structure-activity relationship of this family of molecules. Therefore, we have explored various microbiological and enzymatic methods for the modification of immunomycin. Recently, we reported[8] the synthesis of several desmethylated derivatives of immunomycin by microbial transformation. In addition, we have also isolated and characterized 31-*O*-desmethylimmunomycin O: methyltransferase (DIMT) (Fig. 1) from the immunomycin producing strain[9]. In the present communication, we report the specific methylation at the C-31 hydroxyl group of the multi-desmethylated substrates (13,31-*O*-bisdesmethyl-, 15,31-*O*-bisdesmethyl-, 13,15,31-*O*-tri-desmethyl-immunomycins) using DIMT as enzymatic catalyst and S-adenosylmethionine (SAM) as the methyl donor. The products were purified and identified as 13-*O*-desmethyl-, 13,15-*O*-bisdesmethyl- and 15-*O*-desmethyl-immunomycins. One of these compounds, 13-*O*-desmethylimmunomycin, has previously been reported[8]. The other two, namely, 13,15-*O*-bisdesmethyl-, and 15-*O*-desmethyl-immunomycins have novel structures and were examined for their immunosuppressive activity.

† In previous publications from this laboratory, the name "immunomycin" has been used in place of ascomycin, despite priority in date of publication for the name "ascomycin". In order to avoid further confusion, the name "immunomycin" is still used throughout the present manuscript.

Fig. 1. Reaction catalysed by 31-*O*-desmethylimmunomycin O: methyltransferase (DIMT).



31-Desmethylimmunomycin Immunomycin

Experimental

Enzyme Preparation

DIMT enzyme was isolated and purified from the mycelial cells of *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 55087), an immunomycin producing organism[9].

Substrates Preparation

13,31-*O*-Bisdesmethyl-, 15,31-*O*-bisdesmethyl- and 13,15,31-*O*-tridesmethyl-immunomycins were prepared by biotransformation of the immunomycin according to published procedures[8]. Briefly, immunomycin was biotransformed to the desired desmethylated substrates using a culture of *Actinoplanes* sp. ATCC 53771. The desmethylated products were isolated and purified by HPLC as previously reported[8].

TLC and HPLC Analysis of the Product of the Enzymatic Reaction

The assay was carried out in 1 ml mixture containing 0.025 mM substrate, 2 mM magnesium sulfate, appropriate quantity of the enzyme source in 50 mM phosphate buffer, pH = 7.3. The reaction was initiated by the addition of 1 nmole of ^{14}C-SAM (S-adenosyl-L-[*methyl*-^{14}C]methionine) with a specific activity of 46 mCi/mmol. Incubation of the complete mixture was carried out at 34°C for 20 minutes, and the reaction was terminated by the addition of ethyl acetate. Product of the reaction was extracted with 2 ml of ethyl acetate and 1 ml of the extract was used for TLC analysis. For the analysis of the radioactive reaction product, the ethyl acetate extract was spiked with the standard, when available, and applied to the plastic-supported TLC plate. The plastic sheet was developed in chloroform - methanol (9 : 1) and, after dryness, the UV-light absorbing areas were cut out and the radioactivity was measured with a Beckman liquid scintillation spectrometer (LS 5801) using ScintiVerse I (Fisher Scientific, PA, U.S.A.). In cases where no standard was available, the developed plastic TLC sheet was cut in strips of 0.5 centimeter across the plate and each strip was examined for the presence of radioactivity. HPLC analysis was carried out on the ethyl acetate extract from the enzymatic reaction mixture; the extract was washed with water and dried under nitrogen. The dried residue was dissolved in a small amount of acetonitrile and subjected to the HPLC analysis on a Whatman Partisil 10 ODS-3 (4.6 mm × 25 cm) column. The column was developed either with a linear gradient or an isocratic solvent system. In the linear gradient, the solvent was run for 30 minutes from 45 to 80% acetonitrile containing 0.1% aqueous phosphoric acid. Isocratic separation utilized water - acetonitrile - phosphoric acid (45 : 55 : 0.1) as the mobile phase. In both cases the column temperature was kept at 60°C and 1 ml/minute fractions were collected. A portion of each fraction was examined for the presence the radioactivity.

Large-scale Enzyme Assay and Product Isolation

A typical reaction mixture for the enzymatic synthesis contained 35 mg of partially purified DIMT enzyme; 2 mM magnesium sulfate; 6.25 μM substrate; 60 μM SAM in a total volume of 10 ml which was adjusted with 50 mM phosphate buffer, pH = 7.3. The reaction was initiated by the addition of SAM and incubated at 34°C for four hours. The reaction mixture was extracted with ethyl acetate and the extract was washed with water, dried over sodium sulfate and the solvent was evaporated under reduced pressure. The residue was then taken into acetonitrile and applied on a Whatman Partisil 10 ODS-3 (9.4 mm × 25 cm) column for HPLC purification. This column was developed at 3 ml/minute with a linear gradient solvent system under conditions similar to what was described above for the analytical column. For chemical and biological analysis, fractions isolated from the semi-preparative column was further purified on an analytical column under isocratic or gradient solvent systems as previously described.

Assessment of Biological Activity

A T-cell proliferation assay was used, as previously described[10,11], to examine the immunosuppressive and FK-506 antagonistic activities of immunomycin analogs. Nylon wool purified splenic T-lymphocytes were isolated from C57BL/6 mice and cultured in 96-well flat-bottom microculture plates. Various concentrations of compound or medium alone (control) were added in triplicate wells. The cells were stimulated with ionomycin (250 ng/ml) plus phorbol-myristate acetate (PMA, 10 ng/ml). Cell proliferation was assessed after 48 hours using an MTT colorimetric assay[11]. The results are expressed as percent of the response of cells stimulated in the absence of compound.

FAB-MS and NMR Analysis

Fast atom bombardment mass spectrometry (FAB-MS) was carried out on a MAT 731 instrument and NMR spectra were recorded on a Varian Unity 400 NMR spectrometer.

Results and Discussion

Microbial and enzymatic transformations of known chemicals are convenient means by which new structures can be synthesized. We previously reported[8] the microbial modification of immunomycin (FR-900520) which resulted in the synthesis of three different desmethylated analogs, namely, 13,31-*O*-bisdesmethyl-, 15,31-*O*-bisdesmethyl- and 13,15,31-*O*-tridesmethyl-immunomycins. Recently, an enzyme (DIMT) was isolated which was shown to specifically catalyze the methylation of the C-31-hydroxyl group of 31-*O*-desmethylimmunomycin[9]. The specificity of this enzyme prompted us to examine the above-mentioned multi-desmethylated analogs for 31-*O*-remethylation and, as a result, the following compounds were synthesized and examined for their immunosuppressive activity. Due to the small amount of enzyme available and time requirement in preparation of the substrates by biotransformation methodologies, only 15-*O*-desmethylimmunomycin was made in sufficient amount for the complete structural analysis. The structural assignments for the other two enzymatic products are inferred from the results of the TLC and HPLC elution profiles of the products, and in light of the established substrate specificity of the enzyme[9].

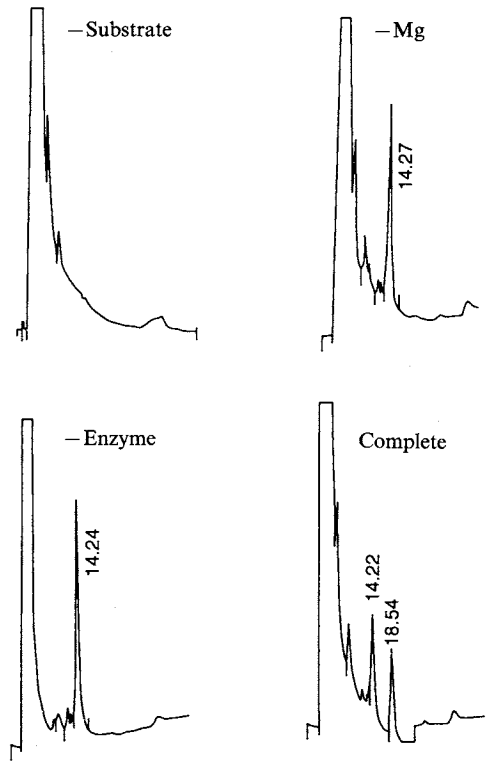
Enzymatic Synthesis of 15-*O*-Desmethylimmunomycin

15,31-*O*-Bisdesmethylimmunomycin was prepared by the biotransformation of immunomycin. This material was used as the substrate in the enzymatic reaction in the presence of DIMT and SAM. The product of the enzymatic reaction was analyzed by a combination of TLC, HPLC, FAB-MS and NMR techniques, as described below.

The results from both TLC and HPLC showed the formation of a new radioactive product indicating transfer of the ^{14}C-methyl group from the labeled SAM to the cold 15,31-*O*-bisdesmethylimmunomycin

substrate. The Rf value of this radioactive enzymatic reaction product on silica gel TLC was greater than that of the substrate (15,31-*O*-bisdesmethylimmunomycin). Furthermore, this material eluted from the reverse-phase HPLC column with a longer retention time than the substrate but shorter than 31-*O*-desmethylimmunomycin. These results were indicative of the specific methylation of the C-31-hydroxyl group and possible formation of the 15-*O*-desmethylimmunomycin. In order to confirm this assignment, a large scale assay was developed in which cold SAM was used as the methyl donor and the product was isolated (Fig. 2, material with the retention time of 18.54 minutes). The FAB-MS analysis of this material showed a molecular ion of 777 atomic mass units (amu) (Fig. 3), which was 14 amu higher than the substrate (763) and 14 amu less than immunomycin (791 amu). The results of the FAB-MS for the isolated enzymatic reaction product in conjunction with its elution time from the reverse-phase column were strong evidence that this material was a mono-desmethyl derivative different from 31-*O*-desmethylimmunomycin. The assignment of the methylation site at C-31-OH rather than C-15-OH of the 15,31-*O*-bisdesmethylimmunomycin substrate was further based on the similarity of the proton chemical shifts of both the enzymatically introduced methyl protons and the C-31 methine proton with their counterparts in standard immunomycin. In immunomycin, the C-13 and C-31 methoxy signals appear close to 3.40 ppm whereas the C-15-methoxy occurs at approximately 3.30 ppm. The chemical shift of the enzymatically introduced methyl signal was determined to be 3.41 ppm. In addition, methylating CHOH generally results in a 0.1 ~ 0.2 ppm upfield displacement of the methine proton. A comparison of the C-31-H chemical shift in 15-*O*-desmethylimmunomycin with the 15,31-*O*-bisdesmethylimmunomycin substrate showed the expected 0.1 ppm upfield shift. Thus, the chemical shift of the new methoxy with the concurrent upfield displacement of C-31-H constituted compelling evidence for enzymatic methylation at C-31-OH of the 15,31-*O*-bisdesmethylimmunomycin.

Fig. 2. HPLC elution profiles (A_{205}) of the product of the enzymatic reaction mixtures under gradient solvent system.

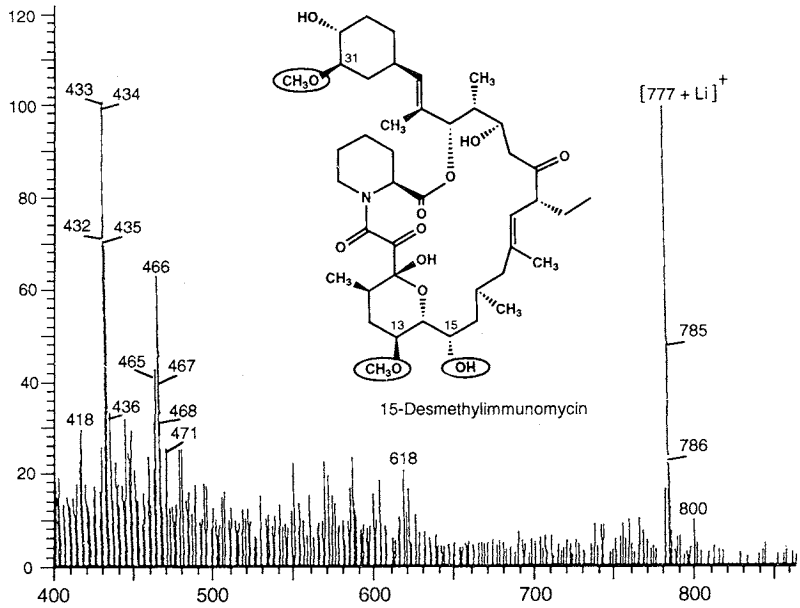


In the complete reaction mixture, 15,31-*O*-bisdesmethylimmunomycin (substrate) and 15-*O*-desmethylimmunomycin (product) are eluted at 14.22 and 18.54 minutes, respectively.

Enzymatic Synthesis of 13-*O*-Desmethyl- and 13,15-*O*-Bisdesmethyl-immunomycins

In experiments similar to that described for the synthesis of 15-*O*-desmethylimmunomycin, 13,31-*O*-bisdesmethyl- and 13,15,31-*O*-tridesmethyl-immunomycins were separately used as substrates in reaction mixtures containing DIMT enzyme and [^{14}C-methyl] SAM. The products of the respective enzymatic reaction mixtures were isolated and analyzed by TLC and HPLC as described above. When

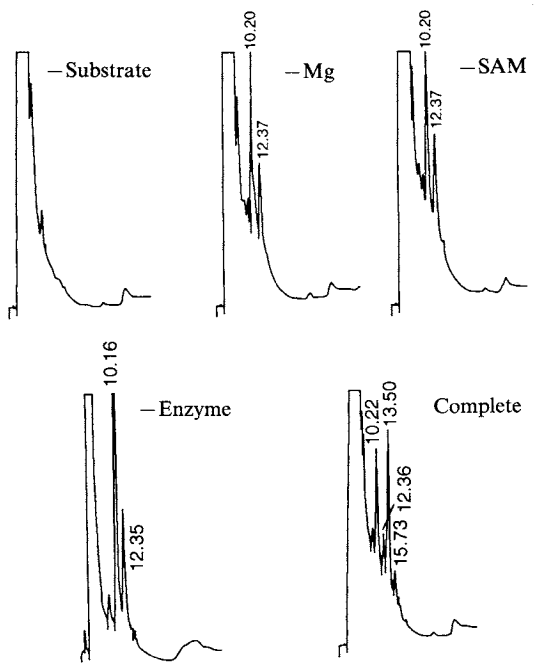
Fig. 3. FAB-MS spectrum of 15-*O*-desmethylimmunomycin.



13,31-*O*-bisdesmethylimmunomycin and ^{14}C-SAM were used as substrate/co-substrate, a radioactive product was isolated having an Rf value and retention time identical to that of 13-*O*-desmethylimmunomycin. The procedures was successfully scaled-up to afford product that was isolated for further analysis (Fig. 4). As shown in the bottom right hand portion of this figure, the product elutes as two peaks with a retention times of 13.50 and 15.73 minutes, identical to that of the standard 13-*O*-desmethylimmunomycin. In the same figure, the elution profile of the substrate is shown which also elutes as two peaks with retention times of 10.22 and 12.36 minutes. Also shown in this figure is the complete lack of product formation when magnesium, SAM, or enzyme had been eliminated from the reaction mixtures. The characteristic two peak pattern observed in the HPLC elution profile of the 13-*O*-desmethylated analogs has previously been discussed[8] and has been attributed to the presence of two non-isolable isomers which differ in the point of hemiketal formation at C-13.

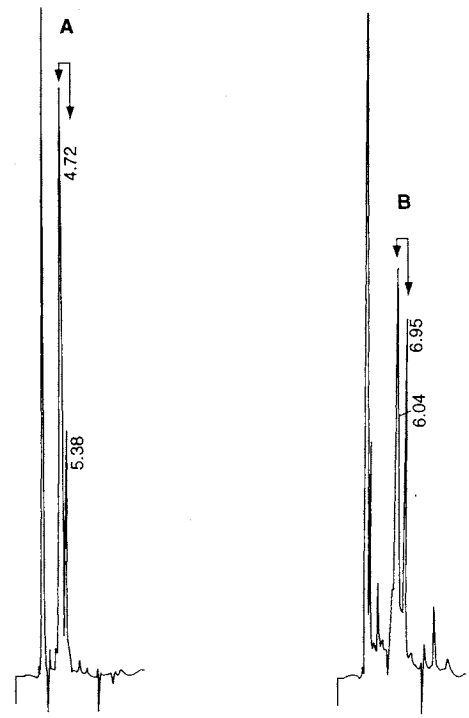
In the work reported here, characteristic two peak pattern of the HPLC profile proved to be useful in characterizing not only 13-*O*-desmethylimmunomycin, as shown above, but also as one of the criteria in the isolation and characterization of 13,15-*O*-bisdesmethylimmunomycin. This latter analog was enzymatically synthesized from the 13,15,31-*O*-tridesmethylimmunomycin using DIMT and SAM, and purified according to the procedures described above for the synthesis of 13-*O*-desmethylimmunomycin. As shown in Fig. 5A, the HPLC profile of the substrate, 13,15,31-tridesmethylimmunomycin, showed the characteristic two peaks pattern with retention times of 4.72 and 5.38 minutes under isocratic solvent conditions. The HPLC profile of the enzymatically methylated product also appeared as two peaks with retention times of 6.04 and 6.95 minutes (Fig. 5B). The two peaks pattern of the HPLC profile is indicative of the presence of the free hydroxyl group at C-13 position, and supports our studies[9] that the enzyme specifically catalyzes the methylation of the C-31 hydroxyl group. This data was further evidence for the assignment of the 13,15-*O*-bismethylimmunomycin as the product of the enzymatic reaction in which

Fig. 4. HPLC elution profiles (A_{205}) of the product of the enzymatic reaction mixtures under gradient solvent system.



In the complete reaction mixture, 13,31-*O*-bisdesmethylimmunomycin (substrate) is eluted as two peaks at 10.22 and 12.36 minutes. The product, 13-*O*-desmethylimmunomycin, is also eluted as two peaks at 13.50 and 15.73 minutes.

Fig. 5. HPLC elution profiles (A_{205}) of the substrate (A) and the enzymatic reaction product (B) under isocratic solvent system.



A is 13,15,31-*O*-tridesmethylimmunomycin, and B is 13,15-*O*-bisdesmethylimmunomycin.

13,15,31-*O*-tridesmethylimmunomycin was used as substrate. Additionally, the assay was carried out with the labeled SAM and the radioactive product was isolated and examined by HPLC. The elution time of the resulting radioactive material proved to be similar to the elution profile of the putative 13,15-*O*-bisdesmethylimmunomycin. As previously mentioned, due to the scarcity of the enzyme and substrate (13,15,31-*O*-tridesmethylimmunomycin), not enough material could be prepared for extensive structural analysis. However, sufficient material was prepared for evaluation of biological activity.

Biological Activity

HPLC purified samples of the enzymatically synthesized 15-*O*-desmethyl- and 13,15-*O*-bisdesmethyl-immunomycins were examined for immunosuppressive activity, as well as for antagonist activity towards FK-506[10], in mouse T-cells activated with ionomycin and PMA. For the purpose of comparison, the biological activities of the precursor compounds, 15,31-desmethyl- and 13,15,31-tridesmethyl-immunomycins, were also examined in parallel. The degrees of inhibition of T-cell proliferation caused by various concentrations of these samples are presented in Table 1. Similar to previous reports[8], 15,31-*O*-bisdesmethylimmunomycin had no detectable immunosuppressive activity. However, it was found to antagonize the immunosuppressive effect of FK-506. The 15-*O*-desmethyl analog, on the other hand, did not inhibit T-cell proliferation at any of the concentrations tested but showed somewhat enhanced antagonist activity compared to 15,31-*O*-bisdesmethylimmunomycin. Moreover, while the 13,15,31-*O*-

Table 1. Immunosuppressive and FK-506-antagonist activities of desmethyl derivatives of immunomycin in ionomycin plus PMA-stimulated T-cells[a].

Immunomycin analog	Concentration (nM)	Effect on T-cell proliferation (% of control response)[b]	
		−FK-506	+FK-506 (0.6 nM)
None		100.0	13.3
15,31-*O*-Bisdesmethylimmunomycin	327.2	100.0	54.9**
	163.6	109.0	31.8
15-*O*-Desmethylimmunomycin	1,285.3	95.4	104.0**
	642.7	107.0	116.0
	64.3	92.5	39.6
	32.1	103.7	25.1
13,15,31-*O*-Tridesmethylimmunomycin	333.3	90.8	11.5
13,15-*O*-Bisdesmethylimmunomycin	65.4	15.9*	11.4
	32.7	44.0	11.6

[a] Mouse splenic T-cells were stimulated with ionomycin (250 ng/ml) plus PMA (10 ng/ml) in the presence or in the absence (control) of compound. The proliferative response of the T-cells was assessed by MTT assay after 48 hours of culture.

[b] The results are expressed as percent of the proliferative response of cells stimulated in the absence of compound. Cultures grown in the absence of FK-506 were used to determine the immunosuppressive activity (*) of the immunomycin analogs. Cultures grown in the presence of FK-506 were used to determine the antagonist activity (**) of the immunomycin analogs.

Table 2. The immunosuppressive activity of 13,15-desmethylimmunomycin in ionomycin plus PMA-stimulated T-cells is reversed by exogenous IL-2[a].

Compound	Concentration (nM)	Effect on T-cell proliferation (% of control response)[b]	
		−IL-2	+IL-2
FK-506	0.6	7.4	90.8
13,15-*O*-Bisdesmethylimmunomycin	65.4	10.0	72.0
	32.7	10.5	90.9
	16.4	25.3	94.0

[a] Mouse splenic T-cells were stimulated with ionomycin (250 ng/ml) plus PMA (10 ng/ml) in the presence or in the absence (control) of compound. Human recombinant IL-2 was added at a concentration of 50 U/ml. The proliferative response of the T-cells was assessed by MTT assay after 48 hours of culture.

[b] The results are expressed as percent of the proliferative response of cells stimulated in the absence of compound.

tridesmethyl analog was neither immunosuppressive[8], nor antagonistic, at the concentration tested, the 13,15-*O*-bisdesmethylimmunomycin analog was found to exhibit potent immunosuppressive activity. This shows the effect of methylation at the C-31-hydroxyl group on the biological activity of immunomycin type molecules. As shown in Table 2, this suppressive effect of 13,15-*O*-bisdesmethylimmunomycin was reversible by exogenous IL-2 indicating that it is due to a specific inhibition of IL-2 production by the T-cells. Therefore, the suppressive or antagonist activities of immunomycin analogs are differentially enhanced by the methylation at the C-13-OH, C-15-OH and C-31-OH.

The suppressive effect of FK-506 on IL-2 gene activation has recently been demonstrated to result

from the inhibition of the enzymatic activity of the calcium/calmodulin-dependent phosphatase calcineurin[12,13]. To exert this action, the drug needs to form a complex with the cytosolic binding protein, FKBP[14]. In this regard, LIU *et al.*[12], in an attempt to further elucidate the mechanism of action of FK-506-like molecules, have examined several structural analogs, including immunomycin (FR-900520), FR-900523 (C-21-methyl analog) and 15-*O*-desmethylimmunomycin for binding to FKBP, inhibition of transcription driven by the IL-2 gene promoter element, induction of NF-AT, and for inhibition of calcineurin phosphatase activity. The results showed that 15-*O*-desmethylimmunomycin (its method of preparation was not given) was considerably less active than the other two compounds in all the assays tested. Such results are in accord with the observations presented here. In addition, we demonstrate, for the first time, that 15-*O*-desmethylimmunomycin acts as an antagonist of FK-506. This activity is similar to that of compound L-685,818, the C-18-hydroxy derivative of immunomycin[15], which was found devoid of immunosuppressive activity in spite of its ability to interact with FKBP. The authors concluded that the C-18 position of the macrolide ring does not affect the binding to FKBP but plays a role in the interaction with calcineurin. Such a conclusion has been shown to be consistent with NMR and crystallographic studies of the FKBP-FK-506 complex[16]. The present data, along with those of LIU *et al.*[12], therefore, indicate that the methylation of the hydroxyl at C-15 position of immunomycin plays an important role in imparting immunosuppressive activity to the immunomycin. The mechanism by which this methylation affects the immunosuppressive activity of immunomycin molecule is not known. However, mechanisms similar to those that have been proposed for C-18 hydroxylated immunomycin could be envisioned.

In conclusion, we have prepared all possible desmethylated analogs of immunomycin, both through microbial transformation, as previously reported[8] and through enzymatic transformation. These analogs should be useful not only for further structure-activity relationship studies but also as starting materials for the chemical synthesis of other derivatives.

Acknowledgments

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